In re the Application of:

BA HELM, APM WILSON, D MOREIRA-MACHADO, CE PULLAR and A CAMP (assigned to EURO DPC)

Serial no: 09/133,766 (Continuation)

Filed: 8 December 1998 as a continuation of USSN filed 25 November 1993

Title: Allergen/Inflammatory Testing & Diagnosis

Signed: A P M W

Dated: November 4th 2003.

DECLARATION UNDER 37 CFR 1.132

I, A Penelope Wilson declare that my credentials are as listed in the attached curriculum vitae, for which my references are available on request;

I understand that the above patent application, of which I am an inventor is pending before the US Patent and Trademark Office and that a rejection to Claims 44 and 47-54 remains outstanding.

The nature of the Rejection

Claims 44 and 47-54 have been rejected under 35 USC 112 first paragraph as "containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention", ie the rejected Claims relate to a method for which the specification is not enabling.

The Examiner has based his rejection on *Benyon et al*, which he considers teaches that a variety of molecules which are not allergens (ie compound 48/80, poly-L-lysine, substance P, VIP, somatostatin) cause the release of the

mediator histamine from mast cells. ***The Examiner has stated that these substances are not allergens. Thus based on the teachings of Benyon et al., the Examiner has objected that histamine release in itself does not indicate that a substance will also cause IgE mediated allergic reactions.***

The Examiner has noted that in fact that *Benyon et al* refers to release of histamine by mast cells in response to the aforementioned substances as caused by "non-immunological stimuli". ***The Examiner notes that while Benyon et al. also disclose that specific different specific mediators are released by IgE versus IgE independent mast cell activation, the instant claims do not recite release of any particular mediator. He states that thus the claimed invention could not be used to determine the allergenicity of a substance because nonallergens also cause the release of mediators from mast cells.***

The Examiner states that in fact *Benyon et al* disclose that nonallergens can cause the release of histamine from mast cells in similar amounts to that seen when allergens are used in the screening assay. The claimed invention encompasses a method wherein histamine is the only mediator assayed.

Finally the Examiner has stated that if the agent causes release of mediators from mast cells in the absence of IgE, using the screening assays disclosed in the specification it would not be possible to determine if the agent was an allergen per se (eg capable of inducing IgE antibodies) because the agent causes release of mediators from mast cells in the absence of IgE. ***Therefore that the specification is not enabling for the claimed invention.***

The Examiner accepts the novelty and inventive step of Claims on file.

The basis of this Declaration

In order to show that the specification is enabling for the claimed method I hereby declare, and give detail hereinafter, for the following:

- *Nonallergens: I hereinbelow declare that non-immunological "secretagogues" (compound 48/80, poly-L-lysine, substance P, VIP, somatostatin) are potentially allergenic, and indeed some of these cause symptoms which have the hallmark of an allergic response in addition to having IgE adjuvant activity and would cause a direct or IgE adjuvant mediated allergic response. Therefore mediator release by non immunological secretagogues (Benyon et al) does not contradict the method of the invention, since such substances which the Examiner has termed "non-allergens" have the potential of steering immune responses to bystander antigens, i.e. several mast cell secretagogues induce the release of substances capable of creating a microenvironment which favours the subsequent induction of an IgE isotype.*

- *Controlled Screening Assay: I also declare that the method of the invention relates to an assay, as defined in the amended Claim 44, and that assays of the invention were conducted utilising the appropriate negative and positive controls and reference materials. Known allergens gave rise to mediator release whilst inactivated allergen preparations served as negative controls. In addition a number of mediators were assayed which verified the results and demonstrated that the invention is enabled for more than just histamine release.*

- *The Basis of Screening Assay methods: I also declare that the present invention relates to a method for screening assay of unidentified "virgin" substances the allergenicity of which is not known, to determine allergenicity without the addition of IgE, and should therefore be considered as a first screen. The invention is therefore enabled as the skilled artisan would appreciate that such results may require verification or confirmation. Moreover we are able to tell the difference between an IgE and non-IgE response by the shape of the dose response curve.*

Furthermore one could look at the direct binding of the potential allergen to specific IgE (ie screen serum as I describe below).

- *Non-immunological stimuli capable of causing immunological release: I also declare that the method of the invention is based on the sound findings of our research and more recent research of others which shows that long term or downstream effects of mediator release can cause IgE synthesis, leading to immunological release.*

The detail of this Declaration

Nonallergens

Previous Statements

We have previously submitted in our response dated 16 December 2002 that mediator release by non-immunological secretagogues (*Benyon et al*) does not contradict the method of the invention, since such substances are also capable of steering subsequent immune responses towards an allergy. The Examiner has rejected this submission, in the form of various statements regarding what is known in the art and experimental results that have been obtained, under MPEP Section 716.01 © (Rev. 1 Feb 2003) as Attorney arguments which cannot take the place of evidence.

The rejected statements are my own statements and are based on my extensive experience in this field. I hereby resubmit those statements as follows:
Benyon et al in fact support the viability of this invention. The method of our invention is looking for substances which cause non-immunological mediator release and therefore *Benyon et al's* results support the present invention, although *Benyon et al* did not recognise the significance of their observation in relation to potential allergenicity, since the molecular mechanisms leading to the development of allergic responses were less well defined at the time their observation was made. *Benyon et al* deduced that the mediator release was due

to an incidental factor such as the closeness of some mast cell populations to nerve endings. In fact the skilled person would not at the time, on reading *Benyon et al*, have appreciated that the release observed was an indication of potential allergenicity, either directly or mediated by an IgE adjuvant activity.

The skilled person, on reading *Benyon et al* and being equipped with the knowledge and understanding which has been built up since 1989 by progress in this field, but particularly in view of the knowledge built up from my and my co-inventors own work leading to our invention, would appreciate that *Benyon et al* had in fact observed non-IgE mediated release, which he has termed non-immunological, and which may be linked to potential allergic responses in individuals. Our invention therefore shows that substances have been observed in the past, for example by *Benyon et al*, to induce release of then-unrecognised mediators, which have since been classified as mediators, and the substances classified as allergens whose effect is direct or is mediated by IgE adjuvant activity.

In fact there are now several hundred such mediators including isoforms, and there could well be more mediators or pro-allergenic mechanism, which await identification. In our method, and this was initially the surprising observation, the mediators released by the potential allergens and environmental pollutants which we investigated, like cigarette smoke or diesel exhaust particles, which have IgE adjuvant activity to bystander antigens, which are now in the absence of IgE sensitisation are the same as those released as a result of an IgE-mediated antigenic stimulus. Although we did not look at enough mediators to say that they are the same, and indeed *Levi-Schaffer et al* have investigated differences, we were able to show that at least some of the same mediators are released by at least some of the same substances.

It is now known that people can get stress or heat or cold induced asthma attacks, allergies such as sensitivity to cigarette smoke or diesel exhaust

particles etc., which are not IgE mediated, and it is therefore possible to get the incredible cascade of mediators leading to anaphylactic shock. It is also known that there are very potent substances found within the human body, which substances can cause release *in vivo* and *in vitro*. The present invention in fact aims to detect potential allergens, which are found outside the human body. However the present invention also has the potential of detecting and assessing mast cell activation by endogenous substances. It is known that a mammalian organism in response to external stimuli such as stress produces such substances. It is now known that endogenous cellular mediators, released by activated mast cells (e.g. mast cell proteases) or triggered eosinophils (e.g. rantes) induce mast cell degranulation and cause symptoms of allergy via a non-IgE-mediated mechanism. The present invention represents the first instance that this was appreciated.

Benyon et al in fact investigated substances, which caused release in the human body, and which correspond to the sort of substances, which we would be looking for, using the method of our invention.

New statements

My coinventor Helm was aware of the *Benyon et al* reference at the filing date of this application. Indeed other papers exist and predate *Benyon et al* and disclose a vast number of substances capable of triggering mast cell release in the absence of sensitisation, many of which are in fact allergens.

Benyon et al does not state that the subject non-immunological "secretagogues" are not allergens. At page 898 column 1 he states that "skin mast cells may be activated *in vivo* by cross-linkage of their IgE-receptors. However this is not the only mechanism by which these cells may be activated.... skin mast cells secrete histamine in response to a variety of non-immunological secretagogues including compound 48/80, poly-L-lysine, substance P, vasoactive intestinal

peptide (VIP) and somatostatin". He reports that the IgE dependent and non-immunological stimuli differ in their capacity to activate release of eicosanoids and suggests that the two stimuli use different secretory mechanisms. He states at page 902 column 2 that "the physiological implications of these findings are uncertain but are worthy of consideration... immunological and non-immunological stimuli may have bi-functional roles in the dermis." *Benyon et al* goes on to speculate a homeostatic function of non-immunological stimuli such as neuropeptides.

In my opinion were the method of our invention performed with exposure of cells as described to the non-immunological substances of *Benyon et al*, the method would result in mediator release and a determination that these substances are potential allergens whose effect is either direct or is mediated by an IgE adjuvant activity. This would support my opinion that the substances may indeed be potential allergens, but in fact as many are extremely powerful agents - some of the venoms would probably cause death before they had the chance to evoke an allergic response – their status as allergens has not been recognised *in vivo*. Indeed scorpion venoms are related to bee/wasp/hornet allergens but are so powerful that they could, as a toxin, kill an individual, before initiating an allergic response. However, it is noteworthy that classical allergic responses, and indeed death from anaphylactic reactions in response to repeated scorpion stings have been recorded. The skilled person would without doubt treat these potent substances with care and avoid direct contact. Accordingly the classification of such secretagogues by the Examiner as non-allergens is unfounded and is certainly not established in the literature. I therefore consider that the Examiner has based his objection on an unfounded premise, and that the factual verification of this is yet to be established in the art.

It is well known that some substances do not cause mediator release in low doses but above a certain dose can cause release, and this further supports the

idea that self proteins may not elicit an allergic response in low dose *in vivo* but may steer subsequent allergic responses to bystander antigens if presented to a subject or to mast cells in higher doses or if presented differently (ie could have problems with mast cells when inhaled).

The Examiner has noted that Benyon has in fact determined that specific different specific mediators are released by immunological and non-immunological stimuli. In my opinion this is irrelevant to the matter in hand. The art (*Levi-Schaffer et al, Komisar et al* previously referred) documents different release mechanisms, different release levels, different release promoters and so on, and it is clear that there is much diversity in responses to different substances, presented in different doses, singly or repeatedly presented etc. This is borne out by the clinical observation that each allergy and each exposure incident may be different from the next in severity or response, onset of allergy and disappearance of allergy in older or younger subjects and the like.

In fact in the method of the invention the cells could potentially release any mediator, to a greater or a lesser extent, with which it was preloaded by virtue of the degranulation event caused by a potential allergen or e.g. environmental pollutants with IgE adjuvant activity.

Controlled Screening Assay

New statements

I draw the Examiners attention to the fact that, as mentioned above, the present application includes examples in the form of methods which have been conducted with appropriate controls, in order to eliminate the instance of such misinterpretation of results. I refer the Examiner to Example 1 at Page 15 lines 6 to 13 and Example 2 at page 16 lines 16 to 21 which provide an Example of incubating transfected cells which had been preloaded with mediator, with

serum from an individual known to be sensitised to bee venom, and challenging with bee venom. A dose response curve was obtained as shown in Figure 3 and Figure 4, confirming that release of mediators (5-HT) is an indication of IgE mediated allergic reaction in a sensitised individual. This example acts as a reference for Example 2 at page 16 lines 21 to 25, in which transfected cells preloaded with mediator (5-HT) were challenged with bee venom in the absence of sensitising serum and showed release of mediator 5-HT.

Significantly the Figure 4 shows a different relation of dose and response for sensitised and non sensitised cells, notably a classical "allergic" "bell shaped response with increasing doses of venom, for sensitised cells, and an increasing response with increasing doses of venom for non-sensitised cells. We respectfully submit that the observations of Benyon *et al* are not inconsistent with the method of the invention, since both show that the levels of mediator released by non-immunological stimuli may differ from those of immunological stimuli but the mediators themselves remain a valid indication of potential allergenicity.

Finally in Example 2 at page 16 line 26 to page 17 line 4 and Table 2, the results of challenging with other test substances are shown, and again these are verified by the above Control examples, indicating that mediator release is an indication of potential allergenicity.

The skilled artisan would appreciate that a negative control may be performed by mutating a potential allergen in a point mutation whereby it retains its overall configuration but is deactivated in terms of activating mediator release. We were the first to report this for the allergenicity of enzymatically active bee venom phosholipase A and not its enzymatically inactive counterpart ("A link between Catalytic Activity, IgE-independent mast cell activation, and Allergenicity of Bee Venom Phospholipase A2" Dudler et al, J. Immunol,

1995, 155:2605-2613). We also conducted just this control which we reported in our paper "Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE", Machado et al Eur J Immunol, 1996, 26: 2972-2980, in which we reported that an essentially inactivated house dust mite preparation caused no release. I refer the Examiner to more recent publications such as "The cysteine protease activity of the major dust mite allergen Der p1 selectively enhances the IgE antibody response", Gough et al, J Exp Med, 1999, 190(2), 1987-902.

The Table 2 of the specification shows that levels of at least two mediators (5-HT (preloaded) and protease(naturally occurring)) are released in proportional amounts for any given substance, in some instances release of a third mediator beta-hexosaminidase (naturally occurring) was also measured. The important factor in the assay method of the invention is that a series of mediators is released by a potential allergen and that anyone of the mediators could be measured as a readout. We would not expect the mediators released by non-immunological and immunological IgE mediated release to be significantly different. If degranulation occurs then it is the contents of the secretory granules that are released. The contents themselves may vary in terms of the relative amount of each mediator depending on what the cell has synthesised – which in turn is dependent on the cellular environment and on what may or may not have been preloaded. It does not necessarily follow, and is not of importance to the determination to be drawn from the assay method, that each mediator plays a role, in vivo, in generating an IgE response to the potential allergen.

Accordingly we respectfully submit that it would be unduly limiting of the scope of the invention to restrict Claim 44 to detection of release of certain mediators only, such as exemplified in the present application.

The Basis of Screening Assays

New statements

I hereby state that the specification describing our invention is enabling for the claimed invention which is designed as a simple screening assay for determining potential allergenicity as indicated by mediator release. Screening assays are intended to simplify and ideally speed up screening of many substances simultaneously or in succession. The screen operates on the principle of a proven assay method which has usually been shown to give a positive or negative indication or other such indication or diagnosis. Screening assays typically produce less than 100% sensitivity and less than 100% specificity but give a rapid means to screen a number of candidates for a specific property or condition, and false positives are routinely eliminated as known in the art.

It is common in the art of clinical chemistry to devise a screening assay to determine a positive grouping of subjects or conditions and subject to further tests performed under more stringent conditions. The method of the invention is no different from such screening assays and is defined as determining a potential allergen and by inference, any substance with IgE-adjuvant activity which is thus capable of mediating an allergic reaction. It would be within the knowledge of the skilled in the art to further investigate the results of the screening assay of the invention and further refine the determination obtained with the assay. We refer to Systemic reviews in health care: "Systemic reviews of evaluations of diagnostic and screening tests", Decks, JJ. BMJ (2001) 323,157-162 "Principles of Screening" Eva, M I, Krvchenia E L, Clinics in Perinatology, 28, (2), 273-278 (2001).

A very well known screening assay is the "Ames" test for potential carcinogenicity and it is well recognised that even this highly established and well reputed test does not give 100% specificity. Similarly the well known and

routinely followed triple test for Downs syndrome is known to be considerably less than 100% accurate.

The method of the invention as described in the specification is conducted with controls as described above in order to verify the results obtained and conclusions drawn. Specifically controls applied in the invention as described in the specification include a test against known positive bee venom, and a test against both fresh and auto-catalytically degraded samples of known positive dust mites, in the latter the old sample being found to be enzymatically inactive and devoid of secretagogue activity. This illustrates:
That the method can produce a positive result in a case known to be positive;
That the method can distinguish between two samples of a known allergen and indicate one sample as being inactive.

The Examiner has questioned whether the method would distinguish allergens from non-allergens. The above control clearly indicates that this is the case for enzymatically inactive dust mite emanations.

We submit that the method of the invention may be complemented with the use of further tests which confirm or reject the findings of the screening assay of the invention, whereby the **potential** allergenic status of the substances may be determined.

Referring to the above comment on screening assays in general, it should be appreciated that a positive result in any screening assay is typically referred for further tests to verify or clarify the result obtained. In this case a further test may comprise repeating the test with detection of interleukins such as IL4 and/or IL-13, confirming that the marker detected is indeed indicative of interleukin release, which is the first stage of an allergic response. The interleukin test is very expensive and would not be realistically conducted in screening a large number of candidate allergens. Failure to verify the result

may lead to repeating the test method with fresh sample or with sample prepared in different solvents for example, which would indicate if the sample was contaminated or in the case of false negative results deactivated.

Thereafter verification may comprise determining the antibody IgE specific for the detected potential allergen and screening blood or tissue samples from blood banks to search for any existing blood sample containing specific for this substance, and indicating that the allergy to that allergen already exists in one or more individuals and that the potential allergen can be upgraded to an allergen as IgE antibodies to it have been created in a human or animal. Thereafter suitable packaging notices may be amended to indicate the chemical as a potential allergen or allergen, or other precautions may be taken to avoid accidental onset of allergic reaction, or in extreme cases the substance may be classified as not for use in environmental applications such as packaging, foods and the like.

A negative result in a screening assay is also typically treated with caution as the result may arise from deactivation of a true potential allergen (see above), as in the case of house dust mite in the present invention, sampling in too low a concentration, or simply from sampling an inactive/ degraded source.

In my opinion the implications of the method of the invention is so significant that were the method of the invention to produce a false positive result, designating a true non-allergen as potentially allergenic, this would be trivial in relation to the alternative situation that the method fails to detect a true positive result, thereby failing to recognise a potential allergen. Any result may be verified or eliminated by further assays as desired, but until the role of non-immunological secretagogues art is fully established in the art these should be treated with caution and indeed treated as potential allergens.

Accordingly the teaching of Benyon *et al* infers a situation in which the method of the invention may detect a substance as potential allergen, when in fact it may prove not to be an allergen. This is not inconsistent with the claims of the application which claim the identification of a **potential** allergen. In this case however it should be appreciated that a positive result obtained with the method of the invention for substances recited in Benyon *et al* <u>would</u> be valid, even though these substances include self-proteins and substances found in the human body. These substances are highly potent and should be treated with great caution and would indeed cause a nasty reaction if brought into contact with mucosal membranes of most individuals.

Non-immunological stimuli are capable of immunological release
New statements

Finally the Examiner has argued that an agent which causes release of mediators from mast cells in the absence of IgE in the method of the invention is <u>not</u> shown to be capable of generating IgE antibodies and causing immunological release as an allergen. It is clear from the Examples and Table 2 that substances tested caused non-immunological release and are well known allergens, such as latex, bee venom etc.

It should be appreciated that IgE antibodies are known to be generated by class switching and the stimulus for class switching leads to onset of sensitisation. It is now documented in the literature that the long term or downstream indirect effects of mediator release have a part to play in triggering class switching. For example *Levi-Schaffer et al* study long term effects of mediator release, and recent work shows that prostaglandins can stimulate IgE production: "Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE secreting cells", Fedyk et al, Proc Natl Acad Sci USA 1996, 93(20), 10978-83; and "Prostaglandin E2

promotes B lymphocyte Ig isotope switching to IgE[1]", Roper et al, J Immunol, 1995, 154(1) 162-70.

Although there are many ways of onset of allergic reactions including progressive exposure and sudden onset, the finding that a substance causes mediator release in the absence of IgE shows that it is capable of becoming an allergen in the future. A substance which fails to cause mediator release on the other hand has no known mechanism by which it can cause production of IgE whereby it would be classed as an allergen and capable of eliciting an allergic response.

CONCLUSION

Accordingly we submit that Benyon *et al* fails to show that the method of the invention is not enabled. Specifically we submit that:

It is not established in the art that "non-immunological secretagogues" are not allergens or potential allergens;

Appropriate controls for the method are enabled and moreover are well known in the art - both positive and negative control results have been indicated and obtained;

The need to verify results of the method would be well known to one skilled in the art of screening assays;

The basis for detecting any mediator as an indicator of potential allergenicity, either directly or mediated by IgE adjuvant activity is enabled in the screening assay of the invention *in vitro*. It does not necessarily follow, and is not of importance to the determination to be drawn from the assay method, that each mediator plays a role, in vivo, in generating an IgE response to the potential allergen.

The general state of the art

I refer the Examiner to statements made by my co-inventor Birgit A Helm, under this heading, in the Declaration dated 16 December 1999 and referencing publications of our work subsequent to this invention, illustrating the nature and diversity of allergy and the issue of **potential** allergenicity, effective directly or mediated by IgE adjuvant activity.

REFERENCES

Levi-Schaffer et al, previously referred and of record

Komisar et al, previously referred and of record

"A link between Catalytic Activity, IgE-independent mast cell activation, and Allergenicity of Bee Venom Phospholipase A2" Dudler et al, J. Immunol, 1995, 155:2605-2613 – referred to in earlier declaration of Helm 14.12.99

"Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE", Machado et al Eur J Immunol, 1996, 26: 2972-2980 – referred in earlier declaration of Helm 14.12.99

"The cysteine protease activity of the major dust mite allergen Der p1 selectively enhances the IgE antibody response", Gough et al, J Exp Med, 1999, 190(2), 1987-902

"Systemic reviews of evaluations of diagnostic and screening tests", Deeks, JJ. British Med Journal (2001) 323,157-162 "Principles of Screening" Eva, M I, Krvchenia E L, Clinics in Perinatology, 28, (2), 273-278 (2001).

"Prostaglandin E2 receptors of the EP2 and EP4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE secreting cells", Fedyk et al, Proc Natl Acad Sci USA 1996, 93(20), 10978-83

"Prostaglandin E2 promotes B lymphocyte Ig isotope switching to IgE[1]", Roper et al, J Immunol, 1995, 154(1) 162-70.

CURRICULUM VITAE

PERSONAL DETAILS

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DATE OF BIRTH	27 February 1958	
NATIONALITY	British	MARITAL STATUS Single

QUALIFICATIONS

1969-1976	Coloma Convent Grammar School, Shirley, Surrey, UK
'O' LEVELS	9
'A' LEVELS	3 (Chemistry, Physics, Pure Mathematics)
1976-1981*	Loughborough University of Technology Loughborough, Leicestershire, UK
DEGREE	BSc (Hons) & DIS in Medicinal & Pharmaceutical Chemistry
DIS DISSERTATION Diploma in Industrial Studies	The Chemotherapy of Neoplastic Disease.
1983-1986 Recipient of Tenovus Scholarship	The Tenovus Institute for Cancer Research The University of Wales College of Medicine Cardiff, UK
DOCTORATE	**Breast Cancer Biochemistry** An Investigation of the Interaction of Agonists and Antagonists with the Purified Oestrogen Receptor.

* Five years taken over 4 year sandwich course as suffered from glandular fever 1978

CAREER SUMMARY

Oct 1989- present EURO/DPC Ltd

EURO/DPC is the European Headquarters of DPC, the worldwide leading independent manufacturer of immunodiagnostic kits, and associated instrumentation, for hospitals, doctor's offices, clinical, veterinary, forensic and research laboratories. Further information may be obtained from DPC's web site www.dpcweb.com

Feb 1998 - present Director, Technology Assessment and Acquisition

In this role I report directly to our Chief Scientific Officer at DPC's Corporate Headquarters in Los Angeles and liaise with the Company's Chairman, President and Board of Directors. My responsibilities include:-

- Assessing the relevance of scientific discoveries and new technologies to the Diagnostic and Health Care Industries. I seek analytes, enabling technologies and instrumentation.
- Areas of focus have included molecular diagnostics, in which I've worked closely with Dr James Watson, Nobel laureate and member of DPC's Board of Directors, oncology and reproductive endocrinology.
- Establishing and updating an extensive international network of contacts of relevance to diagnostics, biotechnology and business development.
- Organising meetings and forging links with universities, hospitals, institutes and technology transfer companies.
- Preparation of proposals, licensing agreements, protocols for collaborative research and clinical trials.

Oct 1995- Jan 1998 Scientific Specialist - Tumour Markers

As part of the International Marketing team for Oncology this role involved:-

- Offering advise to our Distributors and their customers on experimental design, protocol evaluation and data reduction.
- Co-ordinating and managing a series of external projects and clinical trials.
- Writing abstracts, scientific papers, marketing literature, scientific appraisals and proposals.
- Attending meetings and presenting scientific and clinical research in the UK and Internationally.
- A major undertaking in 1996 was the organisation of DPC's first International Tumour Marker Conference entitled "PSA and Prostatic Disease".
- Working closely with our International Product Manager for Cancer and a multidisciplinary team of scientists and other specialists world-wide.
- Identifying key areas in which DPC should become involved, especially in the areas of immunology and oncology. Maintaining an up-to-date knowledge of oncology, especially in breast and prostate cancer, was key to the success of the role.

CAREER SUMMARY (continued)

Other Responsibilities (Oct 1995- Jan 1998) :-

- Management of DPC's ImmuStain product line, including planning and prioritising projects on a short and long-term basis. Overseeing Quality Control and Production as necessary, writing SOPs and answering customer queries and complaints.
- Running EURO/DPC's Tissue Culture Laboratory.
- Acting as a Microbiology Consultant for the Company's validation team to ensure that our products and procedures met with FDA approval.

Jan 1994- Oct 1995 **ImmuStain Project Manager, Head of Tissue Culture and Senior Research Scientist**

- Similar to "Other Responsibilities" above except more of an emphasis on practical work and less on marketing and involvement with distributors.

Jan 1991- Dec 1993 **Senior Research Scientist, Head of Tissue Culture and LINK Scheme Co-ordinator.**

(included a 9 month secondment to Dept. Molecular Biology & Biotechnology Sheffield University)

- Managed all EURO/DPC's collaborations with the Department of Molecular Biology and Biotechnology at Sheffield University.
- Successful in securing a MRC co-operative grant and a DTI/SERC LINK scheme award. The work culminated in identifying a new target for therapeutic intervention to treat and prevent allFolloergies - Patents granted
- Involved in the relocation of EURO/DPC to North Wales, designed and established a new Tissue Culture facility.

April 1990-March 1991	**EURO/DPC Ltd**	**Senior Research Scientist**
Oct 1989-March 1990	**EURO/DPC Ltd**	**Research Scientist**

Oct 1983 - Oct 1989 **The Tenovus Institute for Cancer Research, Cardiff, UK Research Scholar/Post-doctoral Scientist**

Research projects, mainly in breast and prostate cancer, included:

- A study of phyto-oestrogens and their interaction with oestrogen receptor proteins.
- The interaction of agonists and antagonists with the purified oestrogen receptor (basis of PhD thesis).
- The development of auto-anti-idiotypic monoclonal antibodies.
- The biochemistry of glucocorticoid-induced antiphospholipase proteins.

Oct 1981 - Sept 1983 **The Marie Curie Research Institute, Surrey, UK Research Assistant** (Breast and Bladder Cancer)

MANAGEMENT EXPERIENCE

My work has involved the management of a wide range of personnel including, technicians, post-doctoral scientists, administrative staff and contract workers.

Management courses have addressed the following topics:- team roles and team building, the creative process, delegation, communication, financial management, negotiation, presentation skills, marketing and public relations. An in depth analysis of personal aptitude, including psychometric tests along with a more advanced look at team roles and management skills.

Other courses have included:- Business development and strategic planning. Marketing and Sales techniques. Opportunities for growth within the Healthcare Industries.

MISCELLANEOUS

In 1995 was nominated by the Director of the CBI, Wales, for a Women in Business Award and became one of five finalists in the category of Science and Technology. Am a member of a number of professional scientific societies. The use of IT is key to the success of my role, I am computer literate and have used and designed a number of databases.

LEISURE INTERESTS

Playing the piano, listening to music (classical and jazz), dance and the theatre. Also badminton, ski-ing, horse-riding and travel.

Funding: PS is supported by a contract from the Agency for Healthcare Research and Quality to the Southern California Evidence-based Practice Center. The Health Services Research Unit is funded by the Chief Scientist Office of the Scottish Executive Department of Health. The Health Services Research Unit and Centre for Health Services Research are members of the MRC Health Services Research Collaboration.

Competing interests: JMG is a member of the Guidelines Advisory Committee for the National Institute for Clinical Excellence and the methodological adviser to the Scottish Intercollegiate Guidelines Network. MPE is chairman of the Guidelines Advisory Committee for the National Institute for Clinical Excellence. SHW is a member of the US Preventive Services Task Force and other practice guideline panels involved in updating.

1 Woolf SH, Grol R, Hutchinson A, Eccles M, Grimshaw J. Clinical guidelines: Potential benefits, limitations, and harms of clinical guidelines. *BMJ* 1999;318:527-30.
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Systematic reviews in health care

Systematic reviews of evaluations of diagnostic and screening tests

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This is the third in a series of four articles

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Tests are routinely used in medicine to screen for, diagnose, grade, and monitor the progression of disease. Diagnostic information is obtained from a multitude of sources, including imaging and biochemical technologies, pathological and psychological investigations, and signs and symptoms elicited during history taking and clinical examinations.[1] Each of these items of information can be regarded as a result of a separate diagnostic or screening "test." Systematic reviews of evaluations of tests are undertaken for the same reasons as systematic reviews of treatment interventions: to produce estimates of test performance and impact based on all available evidence, to evaluate the quality of published studies, and to account for variation in findings between studies.[2-5] Reviews of studies of diagnostic accuracy involve the same key stages of defining questions, searching the literature, evaluating studies for eligibility and quality, and extracting and synthesising data. However, studies that evaluate the accuracy of tests have a unique design requiring different criteria to appropriately assess the quality of studies and the potential for bias. Additionally, each study reports a pair of related summary statistics (for example, sensitivity and specificity) rather than a single statistic (such as a risk ratio) and hence requires different statistical methods to pool the results of the studies. This article concentrates on the dimensions of study quality and the advantages and disadvantages of different summary statistics for combining studies in meta-analysis. Other aspects, including searching the literature and further technical details, are discussed elsewhere.[6]

Summary points

Systematic reviews of studies of diagnostic accuracy differ from other systematic reviews in the assessment of study quality and the statistical methods used to combine results

Important aspects of study quality include the selection of a clinically relevant cohort, the consistent use of a single good reference standard, and the blinding of results of experimental and reference tests

The choice of statistical method for pooling results depends on the summary statistic and sources of heterogeneity, notably variation in diagnostic thresholds

Sensitivities, specificities, and likelihood ratios may be combined directly if study results are reasonably homogeneous

When a threshold effect exists, study results may be best summarised as a summary receiver operating characteristic curve, which is difficult to interpret and apply to practice

Summary statistics of diagnostic accuracy

Sensitivities and specificities
The rates of correct identification of patients with and without the disease are known as test sensitivity and test specificity, respectively.[7] For a test to be useful at ruling out a disease it must have high sensitivity, and for it to be useful at confirming a disease it must have high specificity

Likelihood ratios
Positive and negative likelihood ratios describe the discriminatory properties of positive and negative test results, respectively.[8] Likelihood ratios state how many times more likely particular test results are in patients with disease than in those without disease. Positive likelihood ratios above 10 and negative likelihood ratios below 0.1 have been noted as providing convincing diagnostic evidence, whereas those above 5 and below 0.2 give strong diagnostic evidence.[9] Likelihood ratios can be directly applied to give probabilistic statements concerning the likelihood of disease in an individual (box)

Diagnostic odds ratios
The diagnostic odds ratio is a convenient measure when combining studies in a systematic review (it is often reasonably constant regardless of the diagnostic threshold) but is difficult to apply directly to clinical practice. The diagnostic odds ratio describes the odds of positive test results in participants with disease compared with the odds of positive test results in those without disease. A single diagnostic odds ratio corresponds to a set of sensitivities and specificities depicted by a receiver operating characteristic curve

Studies of diagnostic accuracy

Studies of test performance (or accuracy) compare test results between groups of patients with and without the target disease, each of whom undergoes the experimental test as well as a "gold standard" diagnostic investigation to ascertain disease status. The relation between the test results and disease status is described using probabilistic measures, such as sensitivity, specificity, likelihood ratios, diagnostic odds ratios (box), and receiver operating characteristic curves (box).

Receiver operating characteristic curves

Receiver operating characteristic curves are used in studies of diagnostic accuracy to depict the pattern of sensitivities and specificities observed when the performance of the test is evaluated at several different diagnostic thresholds. Figure 1 is a receiver operating characteristic curve from a study of the detection of endometrial cancer by endovaginal ultrasonography.[8] Women with endometrial cancer are likely to have increased endometrial thicknesses: few women who do not have cancer will have thicknesses above a high threshold whereas few women with endometrial cancer will have thicknesses below a low threshold. This pattern of results is seen in figure 1, with the 5 mm threshold showing high sensitivity (0.98) but poor specificity (0.59) and the 25 mm threshold showing poor sensitivity (0.24) but high specificity (0.98). The overall diagnostic performance of a test can be judged by the position of the receiver operating characteristic line. Poor tests have lines close to the rising diagonal, whereas the lines for perfect tests would rise steeply and pass close to the top left hand corner, where both the sensitivity and specificity are 1. Receiver operating characteristic plots are used in systematic reviews to display the results of a set of studies, the sensitivity and specificity from each study being plotted as a separate point in the receiver operating characteristic space

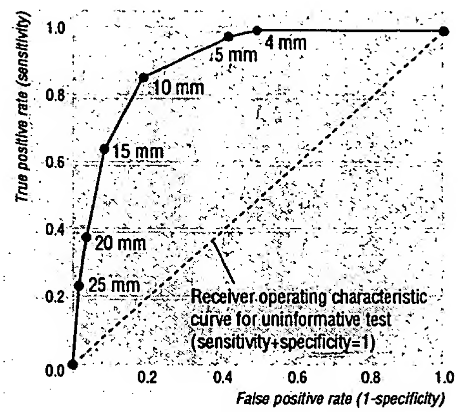


Fig 1 Receiver operating characteristic plot of endovaginal ultrasonography for detecting endometrial cancer

Dimensions of study quality

The quality of a study relates to aspects of the study's design, methods of sample recruitment, the execution of the tests, and the completeness of the study report, as summarised in table 1.[4-6 10-12]

To be reliable a systematic review should aim to include only studies of the highest quality. Systematic reviews may either exclude studies that do not meet these criteria and are susceptible to bias or include studies with a mixture of quality characteristics and explore the differences.[5 5] Whichever approach is adopted, it is essential that the quality of the studies included in the review is assessed and reported, so that appropriately cautious inferences can be drawn.

Empirical evidence

A recent empirical study evaluated which aspects of design and execution listed in table 1 are of most importance.[13] The most notable finding related to the design of the study. Studies that recruited participants with disease separately from those without disease (for example, by comparing a group known to have the disease with a group of healthy controls) overestimated diagnostic accuracy when compared with studies that recruited a cohort of patients unselected by disease status and representative of the clinical population in which the test was used. Studies that used different reference tests according to the results of the experimental test also overestimated diagnostic performance, as did unblinded studies. Omission of specific details from the report of the study was also associated with systematic differences in results.

Meta-analysis of studies of diagnostic accuracy

Meta-analysis is a two stage process involving derivation of summary statistics for each study and

Table 1 Framework for considering study quality and likelihood of bias

Study feature	Qualities sought
Sample of patients	Consecutive or randomly selected sample, recruited as single cohort unclassified by disease state, recruited from clinical setting and point in referral process where test would be used, selection and referral processes fully described, clinical and demographic characteristics fully described, complete
Reference diagnosis	Method and tests described in detail, positive and negative diagnoses clearly described, diagnosis likely to be close to truth, available for all patients, based on same tests and information in all patients, blinding procedures used to prevent knowledge of result of experimental test influencing the reference diagnosis, made before treatment commenced
Experimental test	Application of test described in detail, positive and negative test results clearly described, blinding procedures used to ensure that test is undertaken without knowledge of reference diagnosis, test undertaken before treatment commenced, results reported for all patients, including those with "grey zone" results

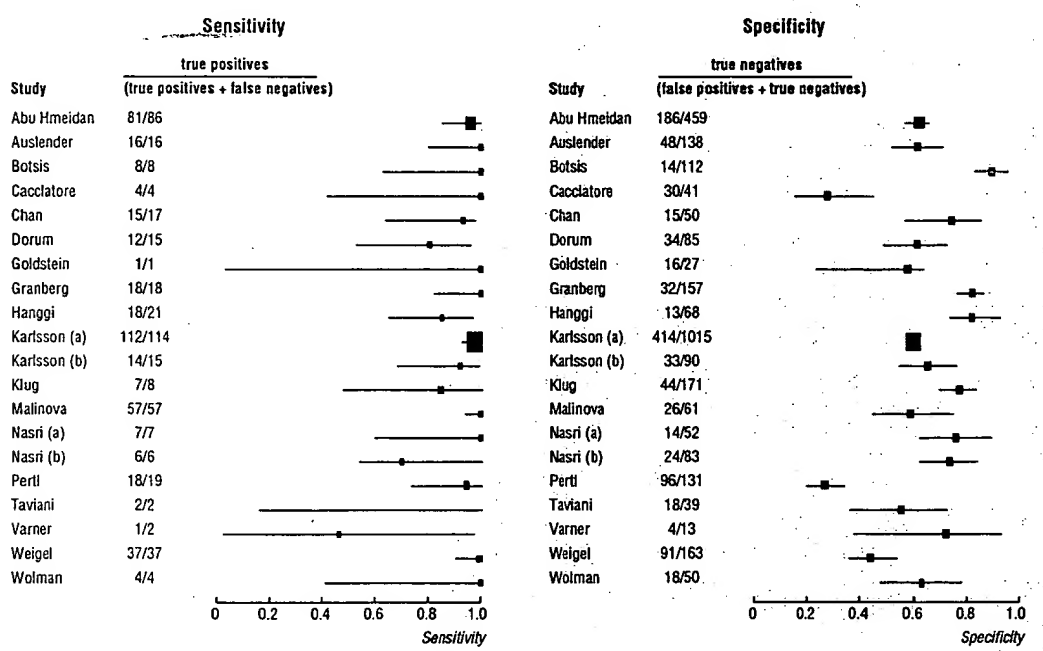


Fig 2 Estimates from 20 studies of sensitivity and specificity of measurement of endometrial thicknesses of more than 5 mm using endovaginal ultrasonography for detecting endometrial cancer.[15] Points indicate estimates of sensitivity and specificity. Horizontal lines are 95% confidence intervals for estimates. Size of points reflects total sample size

computation of a weighted average of the summary statistics across the studies.[14] I illustrate the application of three commonly used methods for pooling different summaries of diagnostic accuracy with a case study.

As with systematic reviews of randomised controlled trials, meta-analysis should be considered only when the studies have recruited from similar patient populations (it is problematic to combine studies from general practice with studies from tertiary care), have used comparable experimental and reference tests, and are unlikely to be biased. Even when these criteria are met there may still be such gross heterogeneity between the results of the studies that it is inappropriate to summarise the performance of a test as a single number.

Case study

Detection of endometrial cancer with endovaginal ultrasonography

Smith-Bindman et al published a systematic review of 35 studies evaluating the diagnostic accuracy of endovaginal ultrasonography for detecting endometrial cancer and other endometrial disorders.[15] All studies included in the review were of prospective cohort designs and used the results of endometrial biopsy, dilation and curettage, or hysterectomy as a reference standard. Most of the studies presented sensitivities and specificities at several endometrial thicknesses detected by endovaginal ultrasonography (the receiver operating characteristic curve in figure 1 is from one of these studies). The case study is based on the subset of 20 studies from this review that considered the diagnostic accuracy of endovaginal ultrasonography in ruling out endometrial cancer with endometrial thicknesses of 5 mm or less. Figure 2 shows the sensitivities and specificities for the 20 studies.

Sources of heterogeneity

The choice of meta-analytical method depends in part on the pattern of variability (heterogeneity) observed in the results. Heterogeneity can be considered graphically by plotting sensitivities and specificities from the studies as points on a receiver operating characteristic plot (fig 3). Some divergence of the results around a central point is to be expected by chance, but variation in other factors, such as patient selection and features of the study's design, may increase the observed variability.[16]

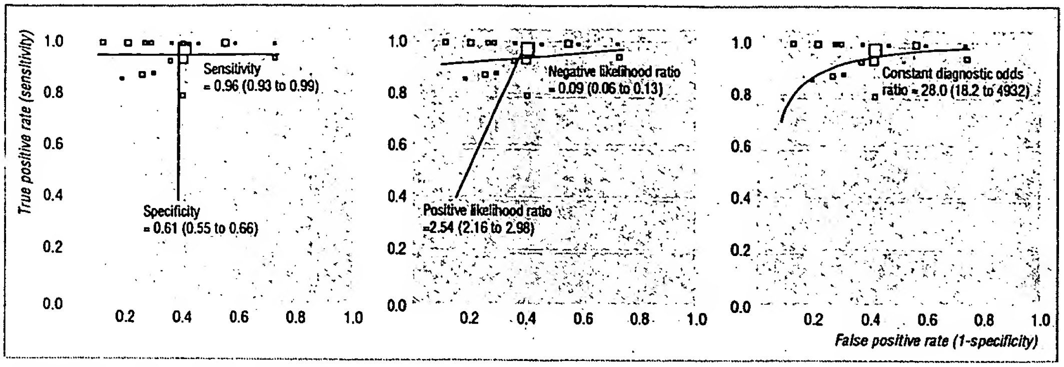


Fig 3 Receiver operating characteristic plots showing three approaches to meta-analysis of 20 studies of diagnostic accuracy of endovaginal ultrasonography for detecting endometrial cancer. Results of studies are indicated by squares. Area of squares is proportional to study sample size. Fitted lines indicate (left) average sensitivity and specificity, (centre) average positive and negative likelihood ratios, and (right) average diagnostic odds ratios. Figures in brackets are 95% confidence intervals for summary estimates

One important extra source of heterogeneity is variation introduced by changes in diagnostic threshold. Studies may use different thresholds to define positive and negative test results. Some may have done this explicitly—for example, by varying numerical cut-off points used to classify a biochemical measurement as positive or negative, whereas for others there may be naturally occurring variations in diagnostic thresholds between observers, laboratories, or machines. The choice of a threshold may also vary according to the prevalence of the disease—when the disease is rare a more extreme threshold may have been used to avoid large numbers of false positive diagnoses. Unlike other sources of variability, variation of the diagnostic threshold introduces a particular pattern into the receiver operating characteristic plot of study results, such that the points show curvature (fig 1).

If there is no heterogeneity between the studies, the best summary estimate of test performance should be a single point on the receiver operating characteristic graph. The first two methods estimate such a summary, first by pooling sensitivities and specificities then by pooling positive and negative likelihood ratios. The third method is more complex and pools diagnostic odds ratios to take account of possible heterogeneity in diagnostic threshold.

Application of a likelihood ratio

The probability of endometrial cancer in a woman with an endometrial thickness of 5 mm or less measured by endovaginal ultrasonography can be computed with Bayes' theorem[1 17]:

Post-test odds = pretest odds×likelihood ratio

Assuming that the study samples are representative, an estimate of the pretest odds can be calculated from the prevalence of endometrial cancer across the studies (13%)

$$\text{Pretest odds} = \frac{\text{prevalence}}{1-\text{prevalence}} = \frac{0.13}{0.87} = 0.15$$

Applying Bayes' theorem to the summary negative likelihood ratio:

Post-test odds = pretest odds×negative likelihood ratio = 0.15×0.09 = 0.014

and converting the post-test odds to a probability:

$$\text{Post-test probability} = \frac{\text{post-test odds}}{1 + \text{post test results}} = \frac{0.014}{1 + 0.014} = 0.013$$

we estimate that only 1.3% of women with an endometrial thickness of 5 mm or less measured by endovaginal ultrasonography will have endometrial cancer. Knowledge of other characteristics of a particular patient that either increase or decrease their prior probability of endometrial cancer can be incorporated into the calculation by adjusting the pretest probability accordingly[1]

Pooling sensitivities and specificities

The pooled estimate of sensitivity is 0.96 (95% confidence interval 0.93 to 0.99) and is depicted by the horizontal line on the receiver operating characteristic plot in figure 3 (left). The overall estimate of mean specificity is lower: 0.61 (0.55 to 0.66).

Heterogeneity is, however, clearly evident in figure 3 (left): although the study points lie reasonably close to the summary sensitivity (test for heterogeneity, $P = 0.04$), the results of many studies lie some distance from the summary specificity (test for heterogeneity, $P < 0.001$).

Regardless of the causes of the heterogeneity, the overall high estimate and relative consistency of the sensitivity results does suggest that a negative test result could be of potential clinical use in ruling out endometrial cancer. As there is heterogeneity between specificities, however, it is more appropriate to note the range of specificities (0.27 to 0.88) rather than to quote the average value of 0.61. It is difficult to draw a conclusion about test specificity: the observed values vary considerably and there is no understanding from this analysis as to the reasons for the variation.

Pooling likelihood ratios

For the case study the pooled estimate of the positive likelihood ratio was not particularly high (2.54, 2.16 to 2.98), and the values varied significantly between the studies (test for heterogeneity, $P < 0.001$). In figure 3 (centre) it is clear that the summary positive likelihood ratio lies some distance from many of the values. Again it is debatable whether reporting the average value of such heterogeneous results is sensible, but it is unlikely that a positive test result could provide convincing evidence of the presence of endometrial cancer as the positive likelihood ratios are all below 10 (data not shown).

The negative likelihood ratios show no evidence of significant heterogeneity (test for heterogeneity, $P = 0.09$), the pooled estimate being 0.09 (0.06 to 0.13), with the summary line on the receiver operating characteristic plot in figure 3 (centre) lying close to the results of most of the studies. This finding again shows

that a measurement of an endometrial thickness of 5 mm or less made by endovaginal ultrasonography can provide reasonably convincing evidence to rule out endometrial cancer.

Although these conclusions concerning potential diagnostic use are similar to those obtained by pooling sensitivities and specificities, the summaries obtained by pooling likelihood ratios can be more easily interpreted and applied to clinical practice. The box describes how the summary negative likelihood ratio can be applied to estimate the probability of endometrial cancer in a woman with a negative test result.

Diagnostic odds ratios and summary receiver operating characteristic curves

If the observed heterogeneity between the studies arises due to variation in the diagnostic threshold, estimates of summary sensitivity and specificity or summary positive and negative likelihood ratios will underestimate diagnostic performance.[18] In this situation the appropriate meta-analytical summary is not a single point in the receiver operating characteristic space but the receiver operating characteristic curve itself. Methods of deriving the best fitting summary receiver operating characteristic curve are necessarily more complex.[2-5 18-21]

How is a summary receiver operating characteristic curve estimated? The simplest approach involves calculating a single summary statistic for each study—the diagnostic odds ratio (box). Each diagnostic odds ratio corresponds to a particular receiver operating characteristic curve. If the studies in a review all relate to the same curve they may have consistent diagnostic odds ratios even if they have variable sensitivities and specificities. Table 2 gives examples of diagnostic odds ratios corresponding to particular sensitivities, specificities, and positive and negative likelihood ratios.

In the case study it is possible that some of the observed heterogeneity could be explained by a threshold effect, perhaps due to differences in calibration of the ultrasound machines. The estimate of the summary diagnostic odds ratio is 28.0 (18.2 to 43.2) and is reasonably consistent across the studies (test for heterogeneity, $P = 0.3$), suggesting that the points indeed could have originated from the same receiver operating characteristic curve. The summary diagnostic odds ratio can be interpreted in terms of sensitivities and specificities by consulting table 2 (for example, a diagnostic odds ratio of 29 corresponds to a sensitivity of 0.95 and a specificity of 0.60 and to a sensitivity of 0.60 and specificity of 0.95) or by plotting the corresponding summary receiver operating characteristic curve (fig 3 (right)). This method does not yield a unique joint summary estimate of sensitivity and specificity: it is only possible to obtain a summary estimate of one value by specifying the value of the other. This greatly limits its clinical application.

Discussion

Systematic reviews of diagnostic accuracy have not, as yet, made the same impression on the practice of evidence based health care as have systematic reviews of randomised controlled trials. Reasons relate to reliability, heterogeneity, and clinical relevance.

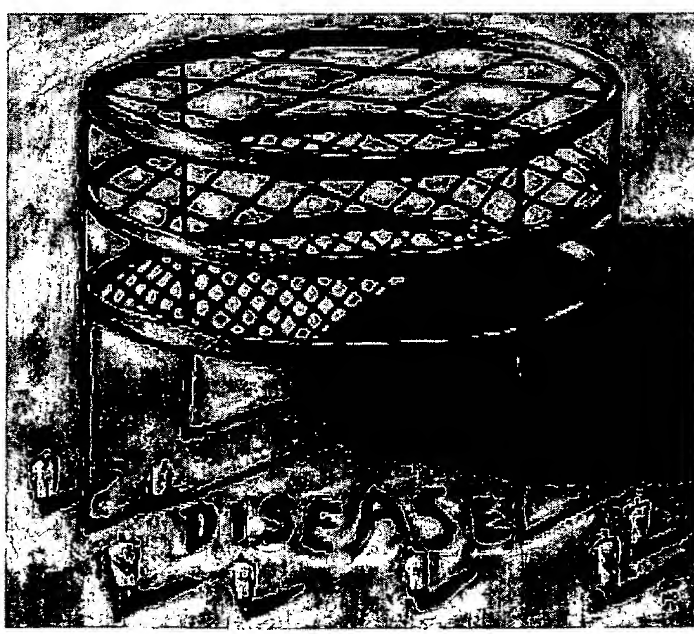
Table 2 Examples of diagnostic odds ratios corresponding to particular pairings of sensitivity and specificity and positive and negative likelihood ratios

	Sensitivity						
Specificity	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**0.95**	**0.99**
0.5	1	2	2	4	9	19	99
0.6	2	2	4	6	14	29	149
0.7	2	4	5	9	21	44	231
0.8	4	6	9	16	36	76	396
0.9	9	14	21	36	81	171	891
0.95	19	29	44	76	171	361	1881
0.99	99	149	231	396	891	1881	9801
Negative likelihood ratio	**Positive likelihood ratio**						
	1	**2**	**5**	**10**	**20**	**50**	**100**
1	1	2	5	10	20	50	100
0.5	2	4	10	20	40	100	200
0.2	5	10	25	50	100	250	500
0.1	10	20	50	100	200	500	1000
0.05	20	40	100	200	400	1000	2000
0.02	50	100	250	500	1000	2500	5000
0.01	100	200	500	1000	2000	5000	10 000

Are systematic reviews of diagnostic studies reliable?

Many meta-analyses of the accuracy of diagnostic tests are hindered by the poor quality of the primary studies: most published evaluations of the accuracy of diagnostic tests having at least one flaw.[12] Headway has been made in understanding the importance of particular features of a study's design and in improving quality, but for many diagnostic tests few high quality studies have been undertaken and published.[13]

The reliability of a review also depends crucially on whether the included studies are an unbiased selection. As with all reviews, systematic reviews of diagnostic tests are susceptible to publication bias, and this may be a greater problem than for randomised controlled trials.[2 5] No investigations, however, have been conducted to estimate rates of publication bias for studies of diagnostic accuracy.



MARK OLDROYD

How useful are systematic reviews to a practising clinician?

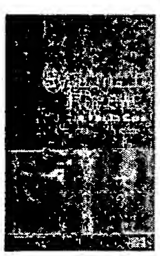
Heterogeneity of the results of studies of diagnostic accuracy is common but in itself does not prevent conclusions of clinical value from being drawn.[22] Despite heterogeneity being observed in the case study, it was still possible to draw a conclusion of clinical value—that an endometrial thickness of 5 mm or less can rule out endometrial cancer.

Diagnostic odds ratios and summary receiver operating characteristic curves are, however, often promoted as the most statistically valid method for combining test results when there is heterogeneity between studies, and they are commonly used in systematic reviews of diagnostic accuracy.[2-4] Unfortunately summary curves are of little use to practising healthcare professionals: they can identify whether a test has potential clinical value, but they cannot be used to compute the probability of disease associated with specific test outcomes. Their use is also based on a potentially inappropriate and untested assumption that observed heterogeneity has arisen through variation in diagnostic threshold. In the case study, whereas the diagnostic odds ratio was a reasonably consistent summary statistic across the studies, there was no evidence to suggest that the observed heterogeneity arose through variations in diagnostic threshold (all included studies had a 5 mm threshold for endometrial thickness). Variation in referral patterns, sample selection, and study methods may be more likely explanations for the heterogeneity. There is no clear statistical advantage in using a summary receiver operating characteristic approach to synthesise the results over pooling sensitivity and specificity or likelihood ratios unless there is a threshold effect. Empirical research is urgently required to find out whether the simpler methods for pooling sensitivities, specificities, and likelihood ratios are likely to be seriously misleading in practice and whether apparent threshold effects are really due to variations in diagnostic threshold rather than alternative sources of heterogeneity.

Are studies of diagnostic accuracy clinically relevant?

Systematic reviews of the accuracy of tests do not always answer the most clinically relevant question. New tests are often evaluated for their ability to replace or be used alongside existing tests. The important issues are comparisons of tests or comparisons of testing algorithms: these would be best addressed in properly designed comparative studies, rather than by synthesising studies of diagnostic accuracy separately for each test.

The evaluation of the diagnostic accuracy of a test is also only one component of assessing whether it is of clinical value.[23 24] Treatment interventions are recommended for use in health care only if they are shown on average to be of benefit to patients: the same criterion should also be applied for the use of a diagnostic test, and even the most accurate of tests can be clinically useless or do more harm than good. It should always be considered whether undertaking a systematic review of studies of diagnostic accuracy is likely to provide the most useful evidence of the value of a diagnostic intervention.



Systematic Reviews in Health Care: Meta-analysis in Context can be purchased through the BMJ Bookshop (www.bmjbookshop.com); further information and updates for the book are available on www.systematicreviews.com.

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Endpiece

Nothing like experience

We live in an age of mass loquacity. We are all writing it or at any rate talking it: the memoir, the apologia, the cv, the cri de coeur. Nothing, for now, can compete with experience—so unanswerably authentic, and so liberally and democratically dispensed. Experience is the only thing we share equally, and everyone senses this.

Martin Amis, *All from experience*,
London: Jonathan Cape, 2000

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Immunology

Prostaglandin E_2 receptors of the EP_2 and EP_4 subtypes regulate activation and differentiation of mouse B lymphocytes to IgE-secreting cells

(cAMP/isotype class switching/FcεRII/major histocompatibility complex class II)

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ABSTRACT **Prostaglandin E_2 (PGE_2) is a potent lipid molecule with complex proinflammatory and immunoregulatory properties. PGE_2 can shape the immune response by stimulating the production of IgE antibody by B lymphocytes and the synthesis of T-helper type 2 cytokines [e.g., interleukin (IL)-4, IL-10], while inhibiting production of Th1 cytokines (e.g., interferon-γ, IL-12). It is unknown what type of receptor binds PGE_2 and modulates these responses. Recent analyses in nonhematopoietic cells have identified six PGE_2 receptors (EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$, and EP_4). This investigation examines quiescent B lymphocytes and reports that these cells express mRNA encoding EP_1, EP_2, $EP_{3\beta}$, and EP_4 receptors. The immunoregulatory functions of each receptor were investigated using small molecule agonists that preferentially bind EP receptor subtypes. Unlike agonists for EP_1 and EP_3, agonists that bound EP_2 or EP_2 and EP_4 receptors strongly inhibited expression of class II major histocompatibility complex and CD23 and blocked enlargement of mouse B lymphocytes stimulated with IL-4 and/or lipopolysaccharide. PGE_2 promotes differentiation and synergistically enhances IL-4 and lipopolysaccharide-driven B-cell immunoglobulin class switching to IgE. Agonists that bound EP_2 or EP_2 and EP_4 receptors also strongly stimulated class switching to IgE. Experiments employing inhibitors of cAMP metabolism demonstrate that the mechanism by which EP_2 and EP_4 receptors regulate B lymphocyte activity requires elevation of cAMP. In conclusion, these data suggest that antagonists to EP_2 and EP_4 receptors will be important for diminishing allergic and IgE-mediated asthmatic responses.**

Prostaglandin E_2 (PGE_2) is a potent lipid molecule that regulates a broad range of physiologic processes in the cardiovascular, endocrine, gastrointestinal, neural, pulmonary, reproductive, visual, and immune systems (1, 2). PGE_2 binds specific protein receptors on a diverse array of target cells. Pharmacologic and cDNA cloning studies have identified six different PGE_2 receptors (EPs), each of which is G protein-associated and belongs to the rhodopsin family of serpentine receptors. Sequences encoding the extracellular domains of EP receptors are highly conserved and each binds PGE_2 with similar affinity. However, the intracellular domains of these receptors are unique. Based on heterogeneous intracellular domains and their association with different intracellular signaling pathways (3), EP receptors are divided into four different subtypes (EP_1–EP_4). EP_1 receptors activate phospholipase C and phosphatidylinositol turnover and stimulate release of intracellular calcium via a poorly characterized G protein-mediated mechanism (3, 4). EP_2 receptors activate adenylate cyclase via a cholera toxin-sensitive, stimulatory G protein ($G_{\alpha s}$) and signal in response to butaprost, an agent (agonist) that selectively binds PGE receptors (3, 5). Molecular analysis of the mouse EP_3 subtype has revealed three different isoforms (α, β, and γ), each of which is an alternate splice variant of the same gene. Sequence differences occur within the 3′ terminus and determine the isoform-specific association with G proteins and characteristic regulatory effects on adenylate cyclase and phospholipase C (3, 7, 8). $EP_{3\alpha}$, $EP_{3\beta}$, and $EP_{3\gamma}$ stimulate release of calcium and inhibit cAMP metabolism via inhibitory G proteins (7, 8). Under certain conditions, $EP_{3\gamma}$ can also stimulate cAMP metabolism via $G_{\alpha s}$ (8). Lastly, EP_4 receptors function similar to EP_2 receptors, activating adenylate cyclase via $G_{\alpha s}$. However, EP_4 receptors are insensitive to the receptor agonist butaprost (3, 6).

PGE_2 is a complex immunomodulator that shifts the balance of the cellular immune response away from T-helper type 1 (Th1) and toward Th2 and drives humoral responses to IgE (2, 9, 10). While lymphocytes themselves do not synthesize PGE_2, nonlymphoid inhabitants of the B-cell microenvironment such as macrophages, follicular dendritic cells, fibroblasts, and vascular endothelial cells produce PGE_2. Moreover, production dramatically increases in response to a variety of immunological stimuli including interleukin (IL)-1, tumor necrosis factor-α, antigen–antibody complexes, and lipopolysaccharide (LPS) (11). Newly synthesized PGE_2 directly regulates activation and differentiation of mature B lymphocytes. For example, PGE_2 inhibits certain activation events such as enlargement and hyperexpression of class II major histocompatibility complex (MHC) and FcεRII (a low-affinity IgE receptor), and it diminishes IgM production. However, PGE_2 dramatically increases production of IgE by synergistically enhancing IL-4 and LPS-induced isotype switching to the ε heavy chain locus (2, 9–14). PGE_2 indirectly modulates humoral responses by modulating the production of cytokines by non-B-lineage cells. PGE_2 profoundly inhibits production of Th1-type cytokines such as, IL-2, interferon-γ, and IL-12 (2, 9, 15, 16). In contrast, PGE_2 does not inhibit Th2 cytokine production, and depending on the mode of T cell activation, can increase production of IL-4, IL-5, and IL-10 (2, 9, 15–17). Thus by enhancing isotype switching to the ε locus and promoting secretion of Th2 cytokines, PGE_2 shifts Ig production to IgE. This relationship is of particular interest because an overproduction of PGE_2 (as high as 10^{-4} M) correlates with elevated Th2 and IgE responses in a number of disorders (AIDS, allergy, hyper-IgE

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Abbreviations: PGE, E-series prostaglandin; EP, PGE receptor; Th1 and Th2, T-helper types 1 and 2; FcεRII, low-affinity IgE receptor; MHC, major histocompatibility complex; LPS, lipopolysaccharide; IL, interleukin; dbcAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate.

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syndrome, Hodgkin lymphoma, sepsis, trauma, and transplantation of autologous bone marrow; refs. 2, 9, 10, and 18).

The complex immunoregulatory effects of PGE_2 are receptor-mediated. Pharmacologic analyses using [^{3}H]PGEs indicate that lymphocytes express a high-affinity receptor that specifically binds PGEs (9, 19–21). Little is known regarding which subtype(s) of EP receptor are expressed by normal lymphocytes (22, 23). Moreover, the diverse immunoregulatory effects of PGE_2 could be explained by heterogeneous patterns of EP receptor expression in hematopoietic cells and tissues. Herein is the first demonstration that primary quiescent B lymphocytes express EP_1, EP_2, $EP_{3\beta}$, and EP_4 receptors. Moreover, this report demonstrates that each subtype contributes differently to immunoregulation by PGE_2.

MATERIALS AND METHODS

EP Agonists and cAMP Reagents. PGE_1, PGE_2, and $PGF_{2\alpha}$ were purchased from Sigma. EP-selective agonists were obtained from a number of sources. 17-Phenyl-ω-trinor-PGE_2 was purchased from Cayman Chemicals (Ann Arbor, MI). Butaprost was provided by M. P. Kotnick (Miles), misoprostol and SC46275 from P. W. Collins (Searle), cicaprost, iloprost, and sulprostone from R. A. Wohl and Berlex Laboratories (Cedar Knolls, NJ). Each subtype of PGE_2 receptor exhibits a unique profile of activation by these agonists (3). The relative potency of each agonist at activating PGE receptor subtypes is: EP_1, $PGE_2 \approx$ 17-phenyl-ω-trinor-$PGE_2 \approx$ cicaprost $\approx$ iloprost > sulprostone; EP_2, $PGE_2 \approx$ misoprostol > butaprost; EP_3, SC4675 > $PGE_2 \approx$ sulprostone > misoprostol; and EP_4, PGE_2 > misoprostol > sulprostone.

Desiccated agonists were reconstituted in absolute ethanol and serially diluted in medium. Agonists were confirmed to be active by the source and were stored according to their instructions. Dibutyryl–cAMP (N^6,$O^{2'}$-dibutyryladenosine-3′,5′-cyclic monophosphate; Sigma), forskolin (Sigma), cholera toxin, SQ22536 (9-{tetrahydro-2-furanyl}-9*H*-purin-6-amine), and RpcAMP (R_p-adenosine 3′,5′-cyclic monophosphothioate triethylamine; Biomol, Plymouth Meeting, PA) were reconstituted in phosphate-buffered saline.

Polyclonal Activators. *Escherichia coli* LPS was purchased from Sigma and recombinant mouse IL-4 from Genzyme. Anti-IgM antibody was rabbit F(ab′)$_2$ anti-mouse IgM (heavy chain-specific) and was obtained from Jackson ImmunoResearch.

Culture Conditions. B lymphocytes were cultured in RPMI medium 1640 (GIBCO) supplemented with 5% fetal bovine serum (HyClone), 5×10^{-5} M 2-mercaptoethanol (Eastman Kodak), 10 mM Hepes (United States Biochemical), 2 mM L-glutamine (GIBCO), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (Sigma), and 50 μg of gentamicin per ml (GIBCO).

Isolation of Quiescent Mouse B Lymphocytes. Rigorously purified, normal mouse B lymphocytes were isolated from spleens of 7- to 22-week-old C57BL/6J × DBA/2J [B6D2(F_1)] male mice (The Jackson Laboratory) using a negative selection and adherence protocol that has been previously described (24). B lymphocytes isolated in this manner are >98% surface IgM-positive, >99% class II MHC-positive, Thy-1.2-negative, nonphagocytic, do not express esterase, and do not proliferate in response to Con A. Purified B cells were fractionated into quiescent and activated subsets via a discontinuous Percoll density gradient (Pharmacia) that was modified to isolate the smallest, most dense B lymphocytes (24). Cells from the lowest interface were harvested and quiescence verified by particle size distribution analysis with a Coulter Channelyzer 256 (24).

Isolation of RNA, Reverse Transcription, and PCR. Total RNA was isolated from cells by the acid guanidinium thiocyanate/phenol/chloroform method (Tri-Reagent, Molecular Research Center, Cincinnati). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primer as previously described (23). For each cDNA synthesis reaction, a parallel reaction was performed without reverse transcriptase (RT−) and the "product" was employed in PCR as a negative control. PCR protocols were specifically tailored for each subtype of EP receptor. Protocols for EP_1, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$, EP_4, and glyceraldehyde-3-phosphate dehydrogenase were performed as previously described (23). EP_2 cDNA was amplified by adding an aliquot of the cDNA synthesis reaction to 10× *Taq* DNA polymerase PCR buffer (Boehringer Mannheim), 1 mM dNTPs, 1 μM oligonucleotide primers (Genosys, The Woodlands, TX) specific for EP_2 [sense (nucleotides 962–982): 5′-TTCGCCATATGCTCCTTGCCT-3′, antisense (nucleotides 1446–1466): 5′-ACGAGACAG-CAACTTGTCTGC-3′], 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim), and water to a total volume of 100 μl. PCR samples were overlaid with mineral oil, initially denatured at 94°C for 2 min and run for 30–40 cycles (94°C for 30 sec, 62°C for 1 min, and 72°C for 2 min) with a final extension at 72°C for 7 min, in a DNA thermal cycler (Perkin-Elmer). Products were fractionated by electrophoresis through a 2% agarose gel containing ethidium bromide. The identity of EP-specific cDNA was confirmed via restriction endonuclease digestion and sequence information using the BLAST sequence comparison program. Sequences were elucidated via dye-terminator cycle sequencing (Applied Biosystems; ref. 23).

Activation Assays. Quiescent B lymphocytes were cultured in flat-bottomed 24-well microtiter plates (Becton Dickinson) at 1×10^6 cells per ml and preincubated with EP agonist or ethanol control for 1 hr. Ethanol controls are cultures that did not receive EP agonist but did receive an equivalent dose of the ethanol solvent in which desiccated agonists were reconstituted. Anti-IgM or LPS and/or IL-4 were added and cultures were incubated at 37°C in a humidified atmosphere with 7% CO_2. Twenty-four hours after addition of LPS and IL-4, B lymphocyte cultures were washed with medium. Cell volumes for both cultures were determined by particle size distribution analysis with a Coulter Channelyzer 256. Cells were analyzed until 200 cells had been recorded in the peak channel (24). Class II MHC and FcεRII expression was monitored via flow cytometry as previously described (12). Briefly, cultures were pelleted and washed in cold PBS containing 0.1% NaN_3 and 1% BSA and incubated at 4°C with saturating concentrations of primary antibody for 1 hr. Class II MHC was detected with supernatant from the M5/114.15.2 hybridoma (rat IgG2b mAb, American Type Culture Collection). FcεRII was detected with the mAb B3B4 (rat IgG2a gift of D. Conrad, Medical College of Virginia, Richmond). After incubation with primary antibody, cells were washed three times and then incubated at 4°C for 1 hr with phycoerythrin-labeled affinity purified goat anti-rat antibody (Fisher). B cells were washed three times and cellular fluorescence was detected with a Coulter Epics Profile (Coulter Electronics) using forward scatter to gate out debris and counting 10,000 events per sample. Irrelevant isotype-matched rat antibody controls (Zymed) and secondary-only (phycoerythrin-labeled goat anti-rat Ig) staining were run as negative controls to establish background fluorescence. For immunofluorescence data, the percentage of B cells in each gate was calculated by CYTOLOGIC software from Coulter.

Proliferation and Differentiation Assays. Quiescent B lymphocytes were cultured at 1×10^6 cells per ml in flat-bottomed 96-well microtiter plates (Falcon; Becton Dickinson) and were stimulated as outlined above for activation assays. For proliferation assays, [^{3}H]thymidine (1 μCi per well; Amersham) was added to each culture 40 hr after addition of mitogen and plates were then incubated for 8 hr at 37°C in a humidified atmosphere with 7% CO_2. Cells were harvested from the cultures with a Packard Micromate 196 harvester and [^{3}H]thymidine incorporation determined via a Packard Matrix 96

direct beta counter. In differentiation assays, supernatants were collected from these cultures 144 hr after LPS and IL-4 stimulation (12). Supernatants were then assayed for the presence of IgM and IgE by the double-determinant ELISA technique in flat-bottomed 96-well microtiter plates (Immulon II; Dynatech) as previously described (14). Standard curves were constructed with known concentrations of mouse IgM and IgE (Sigma).

Viability Assays. In each assay, the viability of B lymphocytes treated with agonist or ethanol control were determined at 24, 48, and 144 hr after treatment by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay from Sigma, as described (24).

Statistics. Data points are the average of quadruplicate cultures. Experiments were repeated between two and five times with similar results obtained in each and representational data are shown (see Figs. 1, 2, 4, and 5). Statistical significance (see Fig. 3) was determined by an unpaired Student's *t* test (two-tailed).

RESULTS

Quiescent B Lymphocytes Express mRNA Encoding the EP_1, EP_2, $EP_{3\beta}$, and EP_4 Subtypes of PGE Receptors. Reverse trancriptase-PCR assays were developed to unambiguously determine which EP receptors are expressed by B lymphocytes. As illustrated in Fig. 1, both quiescent splenic B lymphocytes, as well as the clonal B cell line 70Z/3, yielded reverse trancriptase-PCR products of the anticipated size for EP_1 (501 bp), EP_2 (693 bp), $EP_{3\beta}$ (320 bp), and EP_4 (539 bp). These products were not obtained in reverse transcriptase-negative reactions (data not shown). The identities of these products were confirmed by restriction endonuclease digestion and nucleotide sequence analysis (data not shown). Quiescent B lymphocytes and 70Z/3 do not express mRNA encoding $EP_{3\alpha}$ or $EP_{3\gamma}$ (Fig. 1) despite expressing mRNA encoding other EP receptor subtypes (Fig. 1) and glyceraldehyde-3-phosphate dehydrogenase (data not shown).

EP_2- or EP_2- and EP_4-Selective Agonists Mimic PGE_2-Induced Inhibition of B-Cell Activation. To examine if functional EP_1, EP_2, $EP_{3\beta}$, and EP_4 receptors are expressed and their potential significance in B cell responses, quiescent B lymphocytes were stimulated with EP-selective agonists. These agonists are prostaglandin derivatives that bind specific EP receptor subtypes with higher affinity than other subtypes (3–7, 25, 26). Stimulation of quiescent B lymphocytes with EP-selective agonist only, had little effect on basal expression of class II MHC, FcεRII, or cell volume (data not shown). Stimulation of quiescent B lymphocytes with LPS and IL-4 induces hyperexpression of class II MHC (Fig. 2*A*), by a subset of quiescent B lymphocytes (12, 29). Pretreatment of quiescent B cells with PGE_2 (1×10^{-5} M) almost completely inhibits hyperexpression of class II MHC (92% inhibition, Fig. 2*B*) and FcεRII (data not shown). Pretreatment with butaprost (an EP_2-agonist) or with misoprostol (primarily an EP_2- and EP_4-agonist, some EP_3-activity) also inhibits hyperexpression of class II MHC (95%, Fig. 2*F*; and 97%, Fig. 2*G*, respectively). Viability assays demonstrated that these agonists were not toxic at concentrations up to 1×10^{-4} M (data not shown). Furthermore, agonists that target EP_1 (Figs. 2 *C–E*), EP_3 receptors (Fig. 2 *H* and *I*) or prostaglandin F receptors ($PGF_{2\alpha}$; data not shown) did not inhibit hyperexpression of class II MHC. A similar profile of inhibition was observed when FcεRII expression was monitored (data not shown). Lower doses of PGE_2 or EP-selective agonists also inhibit hyperexpression of class II MHC and FcεRII and inhibition occurs at concentrations as low as 1×10^{-8} M (data not shown). In comparing dose–response profiles for class II MHC and FcεRII expression, EP_2- or EP_2- and EP_4-selective agonists were most effective at inhibiting expression of these activation antigens. The order of agonist potency was: misoprostol ≥ PGE_2 ≥ butaprost ≫ 17-phenyl-ω-trinor-PGE_2 > iloprost = cicaprost = sulprostone = SC46275 = $PGF_{2\alpha}$. Similar results were also obtained when B cells were activated with LPS or IL-4 only (data not shown), or with anti-IgM antibody (data not shown).

Flow cytometric analyses also suggested that PGE_2, butaprost, and misoprostol prevented enlargement (hallmark of transition from G_0 to G_1 in the cell cycle) of B lymphocytes

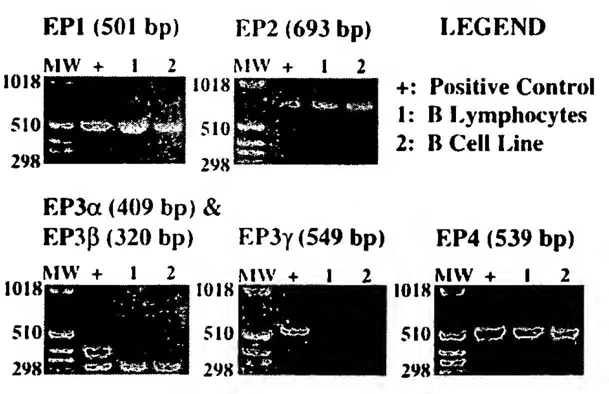

FIG. 1. Resting B lymphocytes express mRNA encoding EP_1, EP_2, $EP_{3\beta}$, and EP_4 subtypes of PGE receptors. Reverse trancriptase-PCR analysis for mRNA encoding EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$, and EP_4. Lane +, positive control cells (P815 mastocytoma − EP_1, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$, EP_4, spleen − EP_2). Lane 1, purified quiescent splenic B lymphocytes; and lane 2, clonal mouse B cell line, 70Z/3. Reverse transcriptase negative reactions did not yield products after PCR (data not shown).

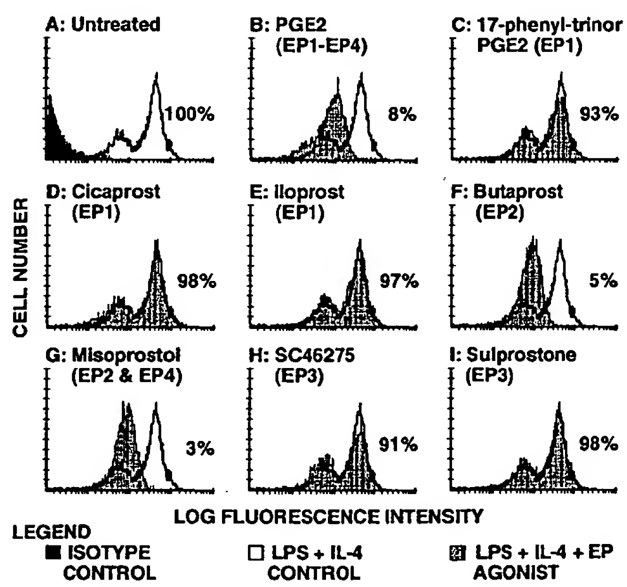

FIG. 2. EP_2- or EP_2- and EP_4-selective agonists mimic inhibition of class II MHC hyperexpression by PGE_2. Flow cytometry detecting class II MHC expression was employed to determine which EP receptors regulate B-cell activation. Quiescent B lymphocytes (1×10^6 cells per ml) were pretreated with a single dose of agonist (1×10^{-5} M for 1 hr) and then received LPS (0.5 μg/ml) and IL-4 (500 units/ml). Twenty-four hours after mitogen stimulation, cultures were stained with a monoclonal anti-class II MHC antibody (M5/114), followed by a phycoerythrin-labeled goat anti-rat antibody (secondary). The percent of cells hyperexpressing class II MHC is indicated in each panel. This percentage was calculated by gating exclusively on the subpopulation of B cells hyperexpressing class II MHC and calculating: [(mean fluorescence intensity of EP receptor agonist, LPS, and IL-4 cultures/mean fluorescence intensity of LPS and IL-4 control cultures) × 100].

stimulated with LPS and IL-4. To accurately measure changes in cell volume, the median volume of cultured B cells was determined with a Coulter Channelizer by cell size distribution analysis. PGE_2, butaprost (EP_2), and misoprostol (EP_2 and EP_4) were equipotent at inhibiting LPS and IL-4-stimulated enlargement over a range of agonist concentrations (Fig. 3 and data not shown). EP_1 and EP_3-selective agonists had no effect (Fig. 3).

PGE_2 also has a mild inhibitory effect on proliferation of B lymphocytes stimulated with LPS and IL-4. On an equimolar basis, the EP_2-selective agonist Butaprost (IC_{50} of 7×10^{-6} M) and the EP_2- and EP_4-selective agonist misoprostol (IC_{50} of 4×10^{-6} M) were as potent as PGE_2 (IC_{50} of 8×10^{-6} M) at inhibiting LPS and IL-4-induced proliferation (data not shown). EP_1-selective agonists and EP_3-selective agonists were dramatically less effective (IC_{50}s $> 5 \times 10^{-5}$ M). At this later time point (48 hr after stimulation with agonist), EP agonist concentrations at 1×10^{-4} M and above decreased the viability of B lymphocytes (data not shown). Overall, the order of agonist potency was: misoprostol = butaprost = $PGE_2 \gg$ sulprostone = SC46275 > 17-phenyl-ω-trinor-PGE_2 = iloprost = $PGF_{2\alpha}$. A similar order of agonist potency was observed for B lymphocytes stimulated with anti-IgM antibody (data not shown).

PGE_2, EP_2-, or EP_2- and EP_4-Selective Agonists Enhance IL-4-Directed Isotype Switching to IgE. Despite inhibitory effects on certain aspects of B-cell activation, PGE_2 enhances cytokine-directed isotype class switching. Pretreatment with PGE_2 enhances production of IgE ≈3.5-fold (Fig. 4*A*) and decreases production of IgM (data not shown) from cultures of B lymphocytes stimulated with LPS and IL-4. On an equimolar basis, the EP_2- and EP_4-selective agonist misoprostol was as potent as PGE_2, while the EP_2-selective agonist butaprost was somewhat less effective than PGE_2 (Fig. 4 *A* and *B*). EP_1-selective agonists modestly increase IgE production (Fig. 4*A*) while EP_3-selective agonists have no effect (Fig. 4*B*). In comparing the overall dose-response profiles for IgE production, the order of agonist potency was: misoprostol ≥ PGE_2 > butaprost > 17-phenyl-ω-trinor-PGE_2 = iloprost = cicaprost > sulprostone = SC46275 = $PGF_{2\alpha}$.

cAMP Mediates the Inhibitory Effect of PGE_2, Butaprost, and Misoprostol. Incubation with PGE_2 increases the level of cAMP 5- to 6-fold in B lymphocytes (27). $PGF_{2\alpha}$, which does

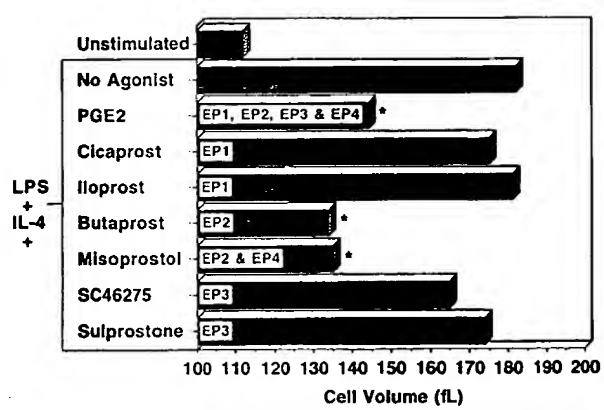

FIG. 3. EP_2- or EP_2- and EP_4-selective agonists mimic inhibition of enlargement by PGE_2. Cell enlargement assay monitoring activation of B lymphocytes. Quiescent B lymphocytes (1×10^6 cells per ml) were pretreated with a single dose of agonist (2×10^{-6} M for 1 hr) and then were treated with LPS (0.5 μg/ml) and IL-4 (500 units per ml). Twenty-four hours after mitogen stimulation, cell volume was determined with a Coulter Channelyzer 256. Data are reported as median cell volume for the culture. Median cell volumes are the mean of three independent experiments and asterisks denote statistical significance ($P < 0.05$) as compared with the no agonist control group.

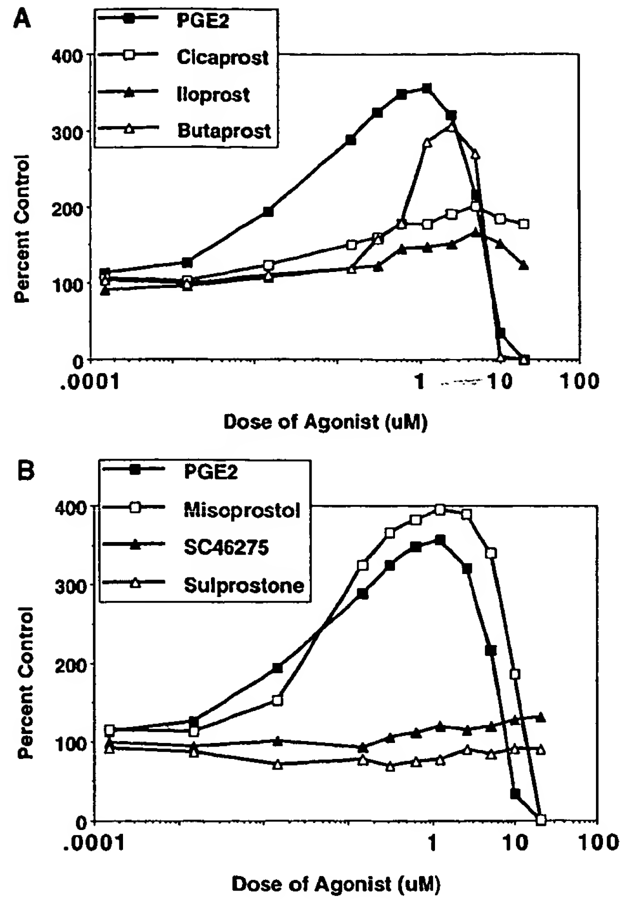

FIG. 4. EP_2- or EP_2- and EP_4-selective agonists mimic enhancement of IgE production by PGE_2. ELISA detecting IgE production by B-cell cultures 144 hr after receiving LPS and IL-4. Quiescent B lymphocytes (1×10^6 cells per ml), were pretreated with a single dose of PGE_2, EP_1, or EP_2 agonist (*A*) or EP_2/EP_4 or EP_3 agonist (*B*) for 1 hr and then received a dose of LPS (0.5 μg/ml) and IL-4 (500 units per ml). Data are reported as percent control response, which was calculated as follows: [(OD for agonist treated cultures)/(OD for control cultures treated with an equivalent concentration of the ethanol vehicle) $\times$ 100].

not bind EP receptors, has no effect (27). Moreover in nonlymphoid cells, activation of EP_2 or EP_4 receptors increases cAMP (3, 5 and 6). To determine whether a cAMP signal mediates the effects of PGE_2, butaprost and misoprostol on quiescent B lymphocytes, cells were treated with agents that manipulate cAMP metabolism. Agents that elevate cAMP, such as cholera toxin (an activator of $G_{\alpha s}$), forskolin (an activator of adenylate cyclase) or dibutyrl-cAMP (dbcAMP, a membrane-permeant analog of cAMP), mimic inhibition of class II MHC hyperexpression by PGE_2, butaprost, or misoprostol (Fig. 5 *B–D*, *G*, and data not shown). To determine if the inhibitory effects of PGE_2, butaprost, and misoprostol require cAMP, we employed SQ22536 (an inhibitor of adenylate cyclase) and RpcAMP (a competitive inhibitor of cAMP-dependent protein kinases) to block cAMP-dependent signaling. Pretreatment with SQ22536 or RpcAMP alone has negligible effects on LPS and IL-4-induced hyperexpression of class II MHC (data not shown). In contrast, PGE_2-induced inhibition of class II MHC hyperexpression (71% inhibition, Fig. 5*D*) is abrogated by pretreatment with SQ22536 (18% inhibition, Fig. 5*E*) or RpcAMP (21% inhibition, Fig. 5*F*). Likewise, inhibition by butaprost (data not shown) or misoprostol (72% inhibition, Fig. 5*G*) was prevented by SQ22536 (17% inhibition, Fig. 5*H*) and RpcAMP (12% inhibition, Fig.

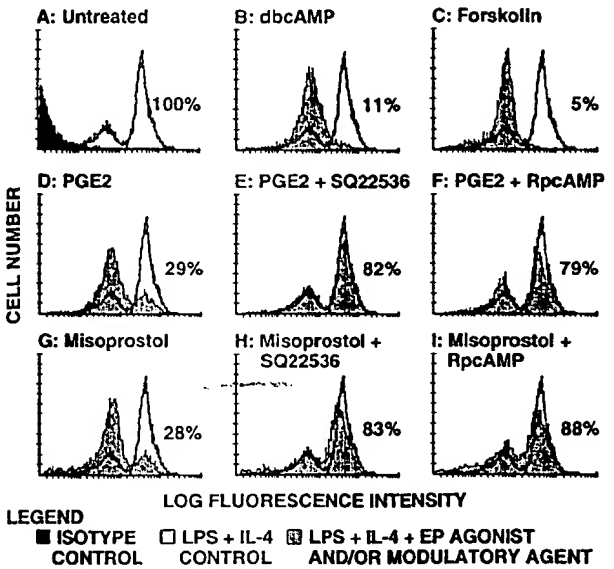

FIG. 5. cAMP mediates the inhibitory effects of PGE_2, butaprost, and misoprostol on class II MHC expression. Flow cytometry detecting class II MHC expression. (*A–C*) Quiescent B lymphocytes (1×10^6 cells per ml) were pretreated with a dose of the cAMP-elevating agent dbcAMP (5×10^{-5} M) or forskolin (1×10^{-5} M) for 6 hr and then with LPS (0.5 μg/ml) and IL-4 (500 units/ml). (*D–I*) Quiescent B cells were initially treated with SQ22536 (1×10^{-4} M), RpcAMP (5×10^{-4} M) or medium control for 1 hr. Cells were subsequently stimulated with PGE_2 or misoprostol (4×10^{-6} M) for 6 hr and then with LPS (0.5 μg/ml) and IL-4 (500 units per ml). In all histograms, cultures were stained for class II MHC 24 hr after mitogen stimulation. As in Fig. 2, the percent of cells hyperexpressing class II MHC is indicated in each panel.

5*I*). In sum, agents that inhibit cAMP metabolism block regulation of B lymphocyte activation by agonists that bind EP_2 and EP_4 receptors.

DISCUSSION

An emerging paradigm is that PGE_2 is a powerful immunomodulator that shifts the balance of the cellular immune response away from Th1 and toward Th2 and drives the humoral response toward IgE (2, 9, 10). Receptors facilitate interaction of PGE_2 with lymphocytes (19–21) and are critical for mediating these immunoregulatory effects. Before this investigation, virtually nothing was known regarding which subtypes of PGE receptors are expressed by normal lymphocytes. A human thymocyte-like line was reported to express EP_2 receptors that regulate apoptosis in response to diverse stimuli (22). We have reported elsewhere that transformed B cells express mRNA encoding EP_1, $EP_{3\beta}$, and EP_4 receptors and that EP_2- and EP_4-selective agonists regulate growth of these transformed cell lines (23). The present investigation is the first to analyze primary, quiescent B lymphocytes and demonstrate that these cells express mRNA encoding the EP_1, EP_2, $EP_{3\beta}$, and EP_4 subtypes of PGE receptors, but do not express $EP_{3\alpha}$ or $EP_{3\gamma}$ receptors (Fig. 1). Reverse trancriptase-PCR analysis of activated B lymphocytes yielded an identical profile of EP receptor subtypes (data not shown).

Coexpression of EP_1, EP_2, $EP_{3\beta}$, and EP_4 receptors indicates that B lymphocytes are capable of responding to PGE_2 in a variety of ways. To determine the immunoregulatory effect each of these receptors mediates, quiescent B lymphocytes were stimulated with EP subtype-selective agonists (3). These agonists have been employed to examine the function of EP receptor subtypes in a variety of nonhematopoietic systems (3–7, 25, 26). This investigation is the first to report that EP_1-selective agonists did not affect activation or proliferation of quiescent B lymphocytes that were stimulated with LPS and/or IL-4, or anti-IgM antibody (Figs. 2 *C–E* and 3 and data not shown). These data illustrate that EP_1 receptors do not regulate these responses. In contrast, the EP_2-selective agonist butaprost and the EP_2-, EP_3-, and EP_4-selective agonist misoprostol dramatically inhibited activation of quiescent B lymphocytes by LPS and/or IL-4 or anti-IgM antibody (Figs. 2 *F* and *G* and 3 and data not shown). The agonist potency ranking for inhibition of B cell activation indicates that both EP_2 and EP_4 receptors mediate this effect. The EP_2-, EP_3-, and EP_4-selective agonist misoprostol is as potent as PGE_2 over a wide range of concentrations, whereas EP_3-selective agonists are ineffective (Figs. 2 and 3 and data not shown), indicating that signaling through both EP_2 and EP_4 receptors is required for mediating the inhibitory effect of PGE_2. While butaprost (EP_2) was as effective as PGE_2 at high concentrations (Fig. 2), it is less effective at concentrations below 1×10^{-5} M (data not shown). These data indicate that EP_2 receptors alone are not sufficient for mediating the overall inhibitory effect of PGE_2. None of the agonists affect hyperexpression of class II MHC or FcεRII by intermediate density B lymphocytes (activated) that were separated from high density (quiescent) B lymphocytes. These B cells were activated *in vivo* (by unknown stimuli) prior to stimulation with PGE_2, Butaprost, or Misoprostol *in vitro*. This indicates that the mechanism by which PGE_2, butaprost, and misoprostol inhibit activation is not reversion of activated cells to a quiescent phenotype. Rather, these agonists prevent events leading to activation. Finally, PGE_2 also has a mild inhibitory effect on proliferation of B lymphocytes stimulated with LPS and IL-4. Butaprost and misoprostol mimic the effect of PGE_2, while the remaining agonists have no effect (data not shown). These data support the conclusion that signaling through both EP_2 and EP_4 is responsible for inhibiting B lymphocyte activation.

Stimulation with IL-4 and LPS is a well-established system for inducing immunoglobulin class switching to IgE (28). PGE_2 enhances this class switch (14). EP_2- or EP_2- and EP_4-selective agonists strongly enhance LPS and IL-4 induced IgE production (Fig. 4 *A* and *B*) while decreasing IgM production $\approx$20%. The EP_2-, EP_3-, and EP_4-selective agonist misoprostol was at least as effective as PGE_2, whereas EP_3-selective agonists are ineffective (Fig. 4*B*), suggesting that EP_2 and EP_4 receptors are sufficient for mediating enhancement by PGE_2. An EP_2-selective agonist (butaprost) was less effective than PGE_2 (Fig. 4*A*), suggesting that EP_2 receptors alone are not sufficient for enhancing IgE production. EP_1-selective agonists only weakly enhanced IgE production (Fig. 4*A*), suggesting that EP_1 receptors are not primarily responsible for mediating enhancement by PGE_2.

Previous investigations examining signaling pathways activated by EP_2 and EP_4 receptors in nonhematopoietic cells reveal that both EP_2 and EP_4 receptors elevate cAMP via activating a $G_{\alpha s}$ protein and adenylate cyclase (3, 5, 6). PGE_2 also elevates cAMP in B lymphocytes (19, 27), and agents that elevate cAMP mimic the effects of PGE_2 on B-cell responses (29). In agreement with these investigations, the cAMP-elevating agents cholera toxin, forskolin, and dbcAMP mimicked the effect of PGE_2, butaprost, or misoprostol on B-cell activation (Fig. 5 *B–D* and *G* and data not shown). To determine if cAMP is required for mediating the effect of EP_2- or EP_2- and EP_4-selective agonists, quiescent B cells were pretreated with agents that inhibit cAMP metabolism. SQ22536 is an inhibitor of adenylate cyclase and blocks the effect of PGE_2, butaprost, or misoprostol on B-cell activation (Fig. 5 *D*, *E*, *G*, and *H* and data not shown). RpcAMP is a nonhydrolyzable competitive inhibitor of cAMP-dependent protein kinases. Pretreatment of quiescent B lymphocytes with RpcAMP also prevented inhibition of class II MHC hyperexpression by PGE_2, butaprost, or misoprostol (Fig. 5 *D*, *F*, *G*,

and *I*, and data not shown). Collectively, these data indicate that PGE_2 binds EP_2 and EP_4 receptors on B lymphocytes, which activates a $G_{\alpha s}$ protein, leading to an increase in cAMP that is required for inhibiting class II MHC hyperexpression. It is also likely that this pathway is responsible for inhibiting hyperexpression of FcεRII and cell enlargement because RpcAMP blocks inhibition of FcεRII expression (data not shown) and cell enlargement (29). It has been reported that inhibition of interferon-γ-induced transcription of the class II MHC and FcγRI loci by PGEs operates by a similar cAMP-dependent mechanism (30). This mechanism also appears responsible for enhancing IgE production because dbcAMP and cholera toxin also mimic the effect of PGE_2 (12, 13, 27). In contrast, the mechanism by which EP_1-selective agonists modestly enhance IgE production (Fig. 4*A*) remains unknown. EP_1 receptors activate phosphatidyl inositol turnover resulting in calcium release in nonlymphoid cells (3, 4). However, calcium mobilization was not observed when B lymphocytes were stimulated with PGE_2 or the EP_1-selective agonist 17-phenyl-ω-trinor-PGE_2 (data not shown). In contrast, stimulation with anti-IgM stimulated a large calcium release (data not shown). While many explanations exist for failing to detect a calcium signal, it is possible that EP_1 receptors stimulate calcium mobilization in B lymphocytes; however, due to low levels of EP_1 expression, this signal may be below the detection limit of the calcium assay.

$EP_{3\beta}$ receptors do not regulate activation, proliferation nor differentiation of B lymphocytes (Figs. 2–4). The presence of mRNA encoding $EP_{3\beta}$ (Fig. 1) suggests that this subtype regulates an activity other than those assayed herein (i.e., activation antigens, cell volume, proliferation and Ig production). While the identity of this activity is unknown, one possibility is that $EP_{3\beta}$ regulates the effects of EP_2 and EP_4 receptors. In contrast to EP_2 and EP_4 receptors, $EP_{3\beta}$ receptors reduce elevated levels of cAMP by activating an inhibitory G protein that interferes with adenylate cyclase activity (3, 7). Therefore, $EP_{3\beta}$ may serve as a regulatory counterbalance by directly antagonizing the cAMP-elevating effects of EP_2 and EP_4 receptors. Thus, the abundance of $EP_{3\beta}$ receptors relative to EP_2 and EP_4 receptors could influence the effect of PGE_2 on B cell activation and differentiation.

The results of this investigation provide new insight as to how PGE_2 regulates the immune response. Binding of PGE_2 to EP_2 and EP_4 receptors elevates intracellular cAMP in B lymphocytes that ultimately promotes differentiation (isotype switching) of these cells, while inhibiting antigen presenting function (class II MHC expression) and clonal expansion (proliferation). Thus, PGE_2 acts as a differentiation agent. It is interesting to note that these results also illuminate a novel mechanism by which LPS may drive B-cell differentiation *in vivo*. Previous data demonstrate that LPS stimulates the production of PGE_2 by monocytes (11) and increases the number of PGE_2 receptors on B cells (19). Additional analysis reveals that LPS increases the amount of mRNA encoding EP_4, but not EP_1 or $EP_{3\beta}$ receptors in B-cell lymphomas (23). Therefore, by stimulating synthesis of PGE_2 and up-regulating expression of EP receptor subtypes that promote differentiation (i.e., EP_4), LPS could drive differentiation and class switching. Although the effect of LPS on expression of EP_2 receptors is unknown, the ability of EP_2 receptors to elevate cAMP (3, 5) and enhance IgE production (Fig. 4*A*) suggests that EP_2 receptors also participate in this novel mechanism. Moreover, this mechanism agrees with other investigations examining the effect of cAMP-elevating agents on IgE production. In these studies, β_2-adrenergic receptor agonists, cAMP analogs, cholera toxin, forskolin, histamine, and phosphodiesterase inhibitors all increase IgE production in mouse and human systems (2, 9, 10, 12, 13) and emphasize the importance of cAMP in regulating IgE production. Therefore, antagonists to EP_2 and EP_4 receptors could emerge as important therapeutics for diminishing allergic, asthmatic, and atopic disorders mediated by IgE.

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The Cysteine Protease Activity of the Major Dust Mite Allergen Der p 1 Selectively Enhances the Immunoglobulin E Antibody Response

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Summary

The house dust mite *Dermatophagoides pteronyssinus* allergen Der p 1 is the most immunodominant allergen involved in the expression of dust mite–specific immunoglobulin (Ig)E–mediated hypersensitivity. The reason for this potent IgE-eliciting property of Der p 1 remains unknown, but there is mounting in vitro evidence linking the allergenicity of Der p 1 to its cysteine protease activity. Here we demonstrate for the first time that immunization of mice with proteolytically active Der p 1 results in a significant enhancement in total IgE and Der p 1–specific IgE synthesis compared with animals immunized with Der p 1 that was irreversibly blocked with the cysteine protease inhibitor E-64. We conclude that the proteolytic activity of Der p 1 is a major contributor to its allergenicity.

Key words: cysteine protease • Der p 1 • IgE

Immunoglobulin E plays a central role in the sequence of events leading to allergic hypersensitivity disorders such as asthma that afflict ~20% of the world population (1). The house dust mite *Dermatophagoides pteronyssinus* allergen Der p 1 has long been recognized as the most immunodominant allergen involved in the expression of dust mite–specific IgE–mediated hypersensitivity (2). The reason for this potent IgE-eliciting property of Der p 1 remains unknown, but there is mounting evidence linking the allergenicity of Der p 1 to its cysteine protease activity (3).

Recent in vitro work has established that Der p 1 selectively cleaves human CD25, the 55-kD α subunit of the T cell IL-2 receptor (the high-affinity form of which consists of α, β, and γ subunits) (4). As a result of cleavage of surface CD25, peripheral blood T cells show markedly diminished proliferation and IFN-γ secretion in response to stimulation by anti-CD3 antibody. It is known that CD4 Th cells undergo a cytokine-driven process of polarization and that IL-2, along with IFN-γ, and IL-4 are considered to be autocrine growth factors for the Th1 and Th2 subsets, respectively (5). The Th1 and Th2 cell populations promote the development of cells of the same subset while suppressing the propagation of those of the other subset. Therefore, Der p 1–induced cleavage of CD25 is likely to lead to impaired growth of cells of the Th1 subset and consequent augmentation of those of the Th2 subset.

These observations raise the question of whether the cysteine protease activity of Der p 1 would bias the immune response in favor of IgE. In this paper, we demonstrate that immunization of mice with proteolytically active Der p 1 results in a significant enhancement in total IgE and Der p 1–specific IgE synthesis compared with animals immunized with Der p 1 that was irreversibly blocked with the cysteine protease inhibitor E-64.

Materials and Methods

Der p 1 Preparation. Der p 1 was isolated from house dust mite fecal pellets (Allergon) by a multistep procedure (6) involving immunoaffinity chromatography on immobilized anti–Der p 1 mAb (clone 4C1; Indoor Biotechnologies), removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (Sigma Chemical Co.), and finally fast protein liquid chromatography (FPLC) to remove low-molecular-mass contaminants. The purity of the preparation was confirmed by NH_2-terminal sequencing on an automatic amino acid sequencer (Applied Biosystems, Inc.), SDS-PAGE analysis (15% gel), and demonstration that enzymatic activity was completely dependent on preactivation with cysteine and totally inhibited by E-64 (L-trans-epoxysuccinyl-leucylamido [4-guanidino]butane). Protein concentration was determined using a bicinchoninic acid (BCA) microtiter plate assay and confirmed spectrophotometrically using the empirical absorption coefficient value for Der p 1 of $E^{1\%}$ (280 nm) = 16.4.

Before use, Der p 1 was preactivated with 5 mM cysteine (Sigma Chemical Co.) to regenerate its thiol group, which becomes oxidized during purification. The catalytic activity of Der p 1 was ascertained in a continuous rate (kinetic) assay using the fluorogenic peptide substrate *N*-tert-butoxy-carbonyl (Boc)-Gln-Ala-Arg-7-amino-4-methyl-coumarin (AMC; reference 6). To block the proteolytic activity of cysteine-activated Der p 1, 1,000-fold molar excess of E-64 (Sigma Chemical Co.) was used; a similar molar ratio of the cysteine protease inhibitor iodoaceta-

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mide (Sigma Chemical Co.) was used as another sulfhydryl reactive agent.

CD25 Cleavage. Spleen T cells were obtained from C57BL/6J mice using standard procedures. The cells (2×10^6) were suspended in RPMI (GIBCO Life Technologies) and stimulated for 3 d at 37°C with Con A (5 μg/ml final concentration) in the presence of IL-2 (100 U/ml) in a humidified atmosphere of 5% CO_2. CD25 cleavage was performed by incubating 10^5 cells with up to 10 μg/ml Der p 1 (preactivated with 5 mM cysteine) for 1 h at 37°C in a total volume of 200 μl serum-free AIM V medium (GIBCO Life Technologies). The cells were then resuspended in RPMI containing 2% FCS, stained for 45 min at room temperature in the dark with FITC-labeled anti–mouse CD25 mAb (clone AMT-13; Sigma Chemical Co.), and fixed with 5% formaldehyde. Cells were analyzed on a FACScan™ (Becton Dickinson) as described (4). The expression of other T cell surface markers, namely CD3, CD4, and CD8, was monitored in the same way using appropriate PE- or FITC-labeled antibodies (clone KT3, Beckman Coulter; and clones YTS191.1 and KT15 [Serotec Ltd.], respectively).

Immunization Protocol. Five groups of 10 female CBA/J mice were given six weekly intraperitoneal injections of 10 μg of proteolytically active Der p 1, 10 μg of E-64–blocked Der p 1, 10 μg of iodoacetamide-blocked Der p 1, 10 μg of OVA (as proteolytically inactive antigen; Sigma Chemical Co.), or 10 μg of OVA with E-64, respectively. All immunizations were given in 200 μg of $Al(OH)_3$ as adjuvant. A tail bleed was obtained 1 wk before the start of immunization (prebleed), and a total bleed was obtained by cardiac puncture 1 wk after the last injection (final bleed). The proteolytic activity of Der p 1 and its inhibition with E-64 or iodoacetamide in the immunization mixture were ascertained as described above.

Antibody Detection. Serum samples were initially titrated to determine the optimal dilution for testing each antibody isotype and subclass. The optimal dilutions used here were 1/10 for detecting total IgE, Der p 1–specific IgE, and OVA-specific IgE and 1/20,000, 1/40,000, and 1/250 for detecting Der p 1–specific IgG, IgG1, and IgG2b, respectively. Total IgE was detected by a sandwich ELISA using one monoclonal anti–mouse IgE (clone R35-72; PharMingen) as capture antibody and a second biotinylated monoclonal anti–mouse IgE (clone R35-118; PharMingen) as a detection antibody. Der p 1–specific IgE, OVA-specific IgE (measured using samples that have been depleted of IgG on a protein G column [Pharmacia]), and Der p 1–specific IgG, IgG1, and IgG2b were detected on microtiter plates coated with a 4 μg/ml solution of either Der p 1 or OVA and developed with biotinylated (for IgE clone R35-118 and IgG1 clone A85-1 [PharMingen] and for IgG2b clone AB275 [The Binding Site]) or alkaline phosphatase–conjugated isotype-specific antibodies (for IgG; Sigma Chemical Co.). Alkaline phosphatase–conjugated Extravidine (Sigma Chemical Co.) was used in conjunction with biotinylated antibodies. Unpaired Student's *t* test was used to compare levels of antibody responses between the different immunization groups; $P < 0.05$ was considered significant.

Results and Discussion

Der p 1 is a 25-kD cysteine protease whose structure has been modeled (7) on the crystal structure of papain, with which it shows considerable sequence similarities, most notably for residues involved in the enzyme active site (8). The proteolytic activity of Der p 1 can be inhibited by E-64, the class-specific inhibitor of microbial origin (9). This inhibition is brought about when cysteine within the Der p 1 active site forms a thioether covalent bond with the epoxy group of E-64. This is an irreversible process that

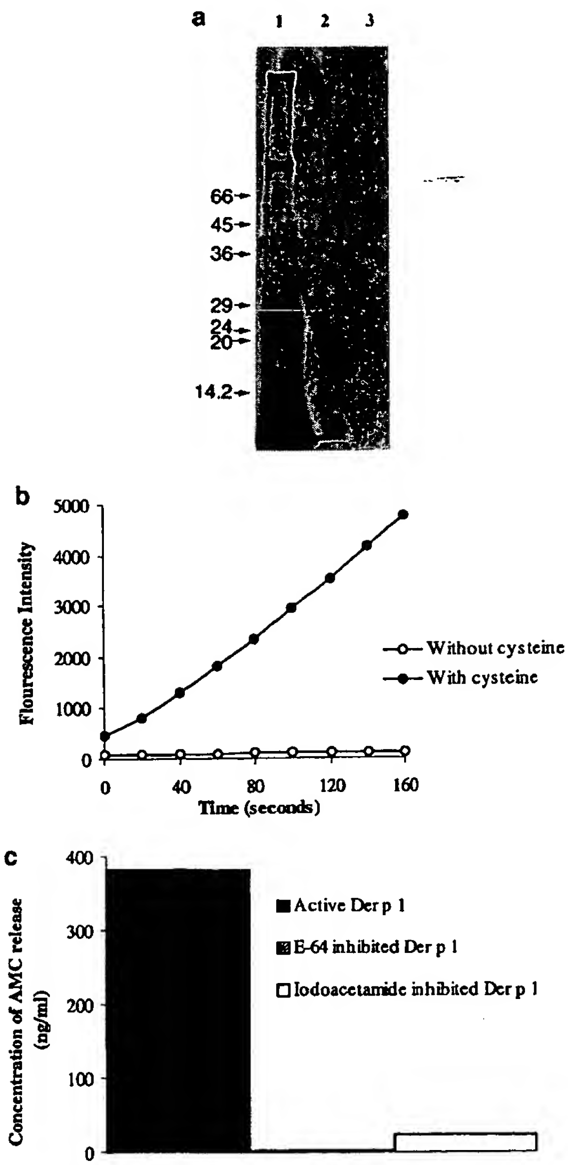

Figure 1. Assessment of the purity of the Der p 1 preparation. NH_2-terminal sequencing showed that the sequence obtained (TNACSINGNA) matches the published sequence of Der p 1 (8). (a) Silver-stained SDS-PAGE analysis showing crude extract of fecal pellets (lane 1), fraction obtained after immunoaffinity chromatography on immobilized anti–Der p 1 mAb 4C1 followed by removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (lane 2), and the final product after FPLC (lane 3). Molecular mass standards are indicated at left. (b) Progression curves of the catalytic activity of Der p 1, measured in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (6), with or without preactivation with cysteine. (c) The addition of E-64 and iodoacetamide results in 100 and 94% inhibition of the enzymatic activity of Der p 1, respectively. Data presented in b and c are the means of duplicate experiments; SE was <5%.

does not lead to significant structural changes, as evidenced by crystallographic studies of a papain–E-64 complex (10).

We have purified Der p 1 from fecal pellets using a multistep procedure and confirmed its purity by NH_2-terminal sequencing, SDS-PAGE analysis, and demonstration that enzymatic activity was completely dependent on preactivation with cysteine and inhibited by E-64 and iodoacetamide (Fig. 1). We have recently shown that Der p 1 selectively cleaves human CD25 from the surfaces of peripheral blood T cells (4). Here we demonstrate that Der p 1 also selectively cleaves CD25 from cultured mouse spleen T cells (Fig. 2), which is not surprising given the high degree of sequence homology that exists between human (11) and mouse (12) CD25. This observation has therefore provided the justification for using this animal species for testing our hypothesis, namely that the proteolytic activity of Der p 1 is a major contributor to its allergenicity.

Intraperitoneal immunization of groups of 10 CBA/J mice with either proteolytically active or inactive (E-64–blocked) Der p 1 over a 6-wk period showed a statistically significant enhancement in total IgE ($P < 0.01$) and Der p 1–specific IgE ($P < 0.02$) responses in animals immunized with proteolytically active Der p 1. This effect was IgE specific, as Der p 1–specific IgG, IgG1, and IgG2b responses increased to the same extent with proteolytically active or inactive Der p 1 (Fig. 3). We are not sure why the IgG1 response did not follow that of IgE, as these two isotypes are considered to be coregulated in the mouse. However, the IgE-restricted enhancement seen in response to immunization with proteolytically active Der p 1 does suggest a mechanism that is unique to IgE isotype switching/synthesis. Fur-

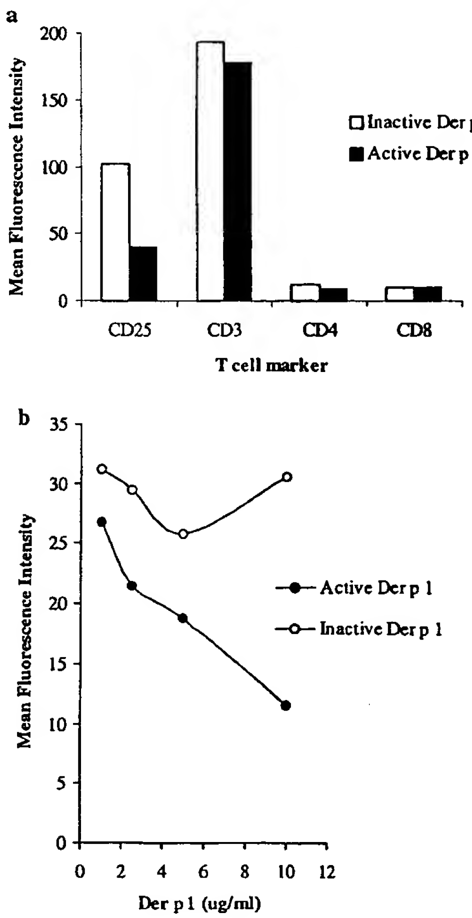

Figure 2. Proteolytically active Der p 1 (10 μg/ml) cleaves mouse CD25, but not CD3, CD4, or CD8, from spleen T cells as monitored by flow cytometry (a); cleavage of CD25 is dose dependent (b). No such effect was demonstrable when Der p 1 was inactivated by E-64. Data presented are the means of duplicate experiments; SE was <5%.

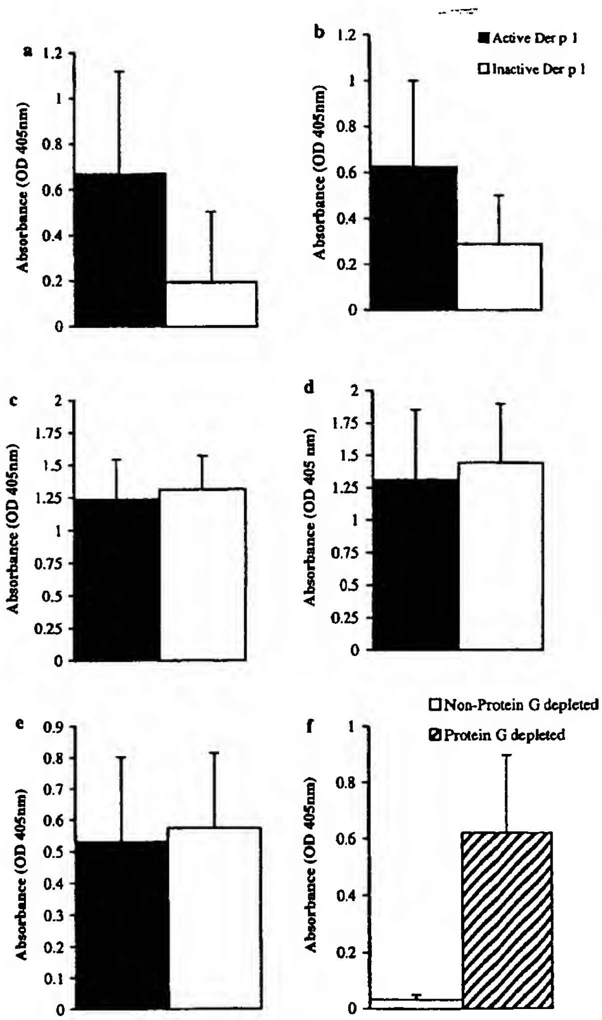

Figure 3. Total IgE (a) and Der p 1–specific IgE (b), IgG (c), IgG1 (d), and IgG2b (e) levels in final bleeds obtained from groups of 10 mice immunized with proteolytically active or inactive (E-64–blocked) Der p 1 measured by ELISA. There was a statistically significant enhancement in total IgE ($P < 0.01$) and Der p 1–specific IgE ($P < 0.02$) responses in animals immunized with proteolytically active Der p 1; prebleeds showed no measurable levels of antibody (except for baseline levels of total IgE). Depleting serum samples of IgG by protein G treatment (to overcome competition) from animals immunized with proteolytically active Der p 1 enhances the detection of Der p 1–specific IgE (f); this effect was also observed with animals immunized with proteolytically inactive Der p 1. Error bars represent 95% confidence intervals, and the results are representative of two immunization experiments.

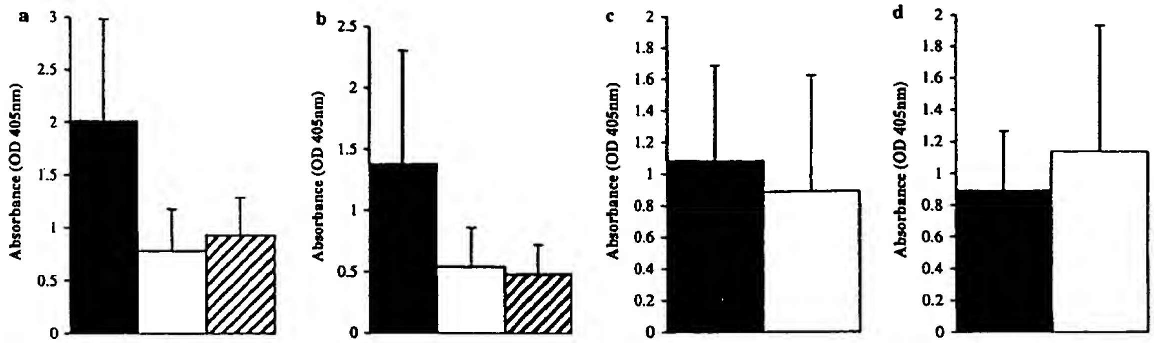

Figure 4. Control experiments showing that the IgE-specific effect observed in Fig. 3 is not due to E-64 exerting a suppressive influence on IgE production by a mechanism that is independent of its binding to the Der p 1 enzyme active site. Total IgE (a) and Der p 1–specific IgE (b) levels in final bleeds obtained from groups of 10 mice immunized with proteolytically active (black bar) or inactive (iodoacetamide [hatched bar]- or E-64–blocked [white bar]) Der p 1 measured by ELISA. There was a statistically significant suppression of total IgE and Der p 1–specific IgE responses in animals immunized with Der p 1 that was rendered proteolytically inactive by either iodoacetamide ($P < 0.04$ for total IgE and $P < 0.05$ for Der p 1–specific IgE) or E-64 ($P < 0.01$ for total IgE and $P < 0.02$ for Der p 1–specific IgE). Total IgE (c) and OVA-specific IgE (d) levels in final bleeds obtained from groups of 10 mice immunized with OVA (black bar) or OVA plus E-64 (white bar) were measured by ELISA. Prebleeds showed no measurable levels of antibody (except for baseline levels of total IgE). Error bars represent 95% confidence intervals.

thermore, our control experiments clearly show that the IgE-specific effect observed here is not due to E-64 exerting a suppressive influence on IgE production by a mechanism that is independent of its binding to the Der p 1 enzyme active site (Fig. 4). First, suppression of total IgE ($P <$ 0.04) and Der p 1–specific IgE ($P < 0.05$) productions was also obtained when the proteolytic activity of Der p 1 was blocked with iodoacetamide, another sulfhydryl reactive agent. Second, the IgE antibody response to OVA, a proteolytically inactive antigen, was not suppressed when the animals were immunized with OVA plus E-64.

Our results are direct evidence that the cysteine protease activity of Der p 1 induces a significant increase in IgE responses. Such an effect is clearly consistent with the ability of Der p 1 to proteolytically cleave mouse CD25 and induce a Th2 response by modulating the balance between IL-4 and IFN-γ (13). The recent demonstration in mice that *Leishmania mexicana* cysteine proteinase–deficient mutants potentiate a Th1 response, compared with the Th2 response normally seen in response to infection with wild-type parasite (14), is also of great relevance here. These findings suggest that immune deviation toward Th1 in dust mite–allergic individuals could potentially be achieved by administering Der p 1 in a catalytically inactive (mutant) form. On the other hand, exploring the potential Th2 adjuvant property of the proteolytic activity of Der p 1 would have important implications in defining principles for modulation of Th1-mediated pathological conditions.

Our demonstration that the cysteine protease activity of Der p 1 enhances total IgE production, apart from increasing Der p 1-specific IgE, suggests that this allergen may play a central role in destabilizing the microenvironment within target tissues to one that is proallergic and thus aids in the initiation and propagation of the allergic cascade. In other words, the proteolytic activity of Der p 1 may exert an IgE-specific adjuvant effect. The in vivo relevance of the proteolytic activity of Der p 1 is further highlighted by reports demonstrating that it increases the permeability of the human respiratory epithelium to macromolecules (15, 16). Such observations, together with our current findings showing a direct effect on the immune system, indicate that the proteolytic activity of Der p 1 is a major contributor to its allergenicity.

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Prostaglandin E_2 Promotes B Lymphocyte Ig Isotype Switching to IgE[1]

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The mechanism by which PG of the E series (PGE) promote murine B lymphocyte IgE production was investigated. We previously reported that PGE, and other agents that increase intracellular cAMP, synergize with IL-4 and LPS to induce IgE and IgG1 production while inhibiting IgM and IgG3 synthesis. These data suggested that PGE may promote IL-4-induced class switching, but the mechanism by which PGE increases IgE synthesis remained obscure. We report here that 1) PGE increases (up to 14-fold) the number of splenic B cells secreting IgE, even though PGE mildly inhibits proliferation. 2) PGE acts on sorted surface IgM positive B cells, consistent with PGE acting on uncommitted B cells to promote class switching to IgE. 3) PGE synergizes with IL-4 to induce germline ε transcripts, demonstrating that PGE acts at the level of transcription in cells that have not yet switched to IgE. 4) In the presence of PGE, rearranged mature V(D)J ε mRNA transcripts can be detected earlier and at higher levels than with IL-4 and LPS alone. Taken together, these data provide strong evidence that PGE synergizes with IL-4 and LPS to direct isotype switching to the ε heavy chain gene in purified B lymphocytes. PGE is a potentially important in vivo immunoregulator, particularly with regard to IgE production and the genesis of allergy. In support of this hypothesis, there are numerous clinical conditions (hyper-IgE, trauma, sepsis, Hodgkin's lymphoma, arthritis) in which overproduction of PGE is coincident with elevated IgE titers.
The Journal of Immunology, 1995, 154: 162–170.

Prostaglandins of the E series (PGE) are secreted by fibroblasts, follicular dendritic cells, macrophages, and epithelial cells (1–5) in response to a vast host of stimuli. For example, PGE is induced by IL-1, IL-6, TNF, LPS, complement components, and cross-linking of Fc receptors (1, 3–8). PGE inhibits numerous immunologic events including B and T lymphocyte proliferation, T cell IL-2 production and IL-2 receptor expression, B cell activation events, and IgM and IgG3 synthesis (9–16). Furthermore, PGE may play a role in inducing tolerance in vivo (17, 18). Recently, however, increasing evidence suggests that PGE is not necessarily a suppressive agent, but is a regulatory one that can enhance certain aspects of the immune response. We previously demonstrated that PGE can promote synthesis of granulocyte-macrophage colony stimulating factor by Th1 cells, and enhances IFN-γ-induced IgG2a production (15, 19). Additionally, we found that PGE_2 and PGE_1, as well as other agents which induce cAMP, enhance IL-4-induced IgE and IgG1 synthesis in supernatants of LPS-stimulated B cells (10, 14, 16, 20). Murine Ag-specific IgE responses are promoted by PGE as well (21), and PGE and cAMP enhance IgE synthesis by human peripheral blood lymphocytes (22). In the presence of PGE, significant quantities of IgE can be produced at much lower concentrations of IL-4. Thus, accessory cell-secreted products can promote the synthesis of certain Ig isotypes by B lymphocytes.

We were particularly interested in PGE regulation of IgE production because of the importance of IgE in human pathology. An estimated 27,000 persons were hospitalized with a primary diagnosis of IgE-mediated disease with an additional secondary diagnosis of 91,000 in 1987 in non-federal hospitals alone (23). It should be noted that these statistics do not include any disease occurring in the out-patient population.

In this paper, we investigate the mechanism of PGE_2 enhancement of IL-4-induced IgE synthesis in vitro, which could be explained by several hypotheses. PGE might stimulate the rate of IgE synthesis while inhibiting production of IgM and IgG3. PGE may alter the kinetics of

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IgE synthesis so that IgE secretion occurs sooner after stimulation, but not in a larger number of cells, or PGE could selectively expand the population of B cells that have already undergone IL-4 directed switching to IgE. Alternatively, PGE might enhance B cell isotype differentiation, increasing the number of lymphocytes that undergo class switching to expression of the Cϵ heavy-chain gene. To determine whether PGE increased the number of IgE-secreting cells, IgE-secreting cells ± PGE were enumerated by ELISA spot assay. To test PGE action on uncommitted B cells, we isolated resting murine splenic B cells expressing surface IgM, as these cells do not secrete IgE (24). These unswitched B cells were then stimulated to Ig synthesis with IL-4 and LPS ± PGE. We show herein that PGE acts on surface IgM-positive B cells and increases the number of cells secreting IgE.

IL-4 induces B lymphocyte class switching to IgE by deletional DNA rearrangement of the C_H locus in both mouse and human (24–33). Before isotype switching, IL-4 induces germline transcripts initiating 5′ of the Cϵ switch region. Induction of germline transcripts upstream of the targeted switch region is a general phenomenon reported for agents that promote switching (25, 28–31, 33). It was therefore important to assess whether PGE augments the pre-switch germline transcripts induced by IL-4 and LPS. Germline transcripts, as well as mature ϵ mRNA, were measured by semi-quantitative PCR (34–36) and Northern analysis. PGE causes an increase in both germline and mature ϵ transcripts compared with controls treated with IL-4 and LPS. Cumulatively, these data demonstrate that PGE promotes class switching to IgE.

Materials and Methods

Isolation of resting B cells

T cell-depleted, Percoll (Pharmacia, Uppsala, Sweden)-purified small dense quiescent B lymphocytes were prepared from spleens of C57BL/6J × DBA/2J (B6D2(F1)), 7- to 20-wk-old, male mice (The Jackson Laboratory, Bar Harbor, ME) as previously described (10, 16). Isolation of quiescent cells was verified by volume analysis after each purification. Lymphocytes prepared in this manner were >95% surface Ig positive as tested using an affinity purified goat $F(ab')_2$ anti-mouse Ig (Cappel, Cooper Biomedical, West Chester, PA). There was no proliferative response to Con A.

Cell culture and experimental protocol

B cells were incubated in RPMI 1640 as previously described (10, 16), with 10% FBS (HyClone, Logan, UT), and 50 μg/ml gentamicin (Life Technologies, Grand Island, NY). For IgE synthesis determination, 5 × 10^5 cells/ml were incubated in triplicate or quadruplicate cultures in 200 ml in 96-well flat-bottom plates (Corning Glass Incorporated, Corning, NY), and for RNA preparation in 15 ml conical tubes (Becton Dickinson, Lincoln Park, NJ) in a humidified 7% CO_2/air mixture at 37°C. Cells were pre-incubated in media alone (as control) or with PGE (Sigma Chemical Co., St. Louis, MO), cholera toxin (List Biologic Labs, Campbell, CA), or dibutyryl cAMP (Sigma Chemical Co.) for 16 h followed by stimulation with *Salmonella typhimurium* or *Eschericia coli* LPS (5 μg/ml) from Sigma Chemical Co. and rIL-4 (generously provided by Dr. W. E. Paul, National Institutes of Health). Hybridomas IGEL b4 and IGEL a2 (American Type Culture Collection, Rockville, MD) at 10^5 cells/ml in 200 μl in triplicate were assayed for the effect of PGE at 10^{-6} M on murine IgE secretion.

Immunofluorescence staining and sorting protocol

Freshly isolated resting B cells were incubated for 30 to 45 min on ice with saturating concentrations of FITC-labeled anti-IgM Ab (μ-chain specific) (Organon Teknika, BCA Cappel, West Chester, PA) and then washed 3 times in PBS. Sterile sorting experiments were conducted on a Coulter EPICS IV (Coulter Electronics, Hialeah, FL) to isolate uncommitted pre-switch B cells (24). Unstained cells were run as negative controls to establish background fluorescence. Sort gates were set to collect the brightest 50% of surface IgM-positive B cells. These cells were analyzed for purity after sorting, and the mean fluorescence intensity was found to be increased 1.5-fold compared with the unsorted population.

ELISA for IgE

Cells secreting IgE were enumerated by a modification of the ELISA protocol for cell supernatants previously described (16). On the appointed day after LPS stimulation, supernatants were removed and B cells were washed and incubated for 2 to 4 h on plates precoated with a rat mAb specific for murine IgE (B1E3, the kind gift of Dr. D. Conrad, Medical College of Virginia). The plate was developed with an affinity-purified (Sigma Chemical Co.) biotinylated (Pierce, Rockford, IL) secondary IgE-specific mAb (R1E4, the generous gift of Dr. M. Kehry, DNAX Research Institute, Palo Alto, CA). After incubation with alkaline phosphatase-labeled streptavidin (Southern Biotechnology, Birmingham, AL) and 5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt (Sigma Chemical Co.), IgE-secreting cells were enumerated as blue spots. Isotype specificity of the reagents was verified with hybridomas secreting murine IgE, IgG1, IgM, IgG2b, and IgG2a.

Polymerase chain reaction primers

Primers were designed to amplify either germline ϵ RNA or mature ϵ V(D)J mRNA (University of Rochester, Department of Microbiology and Immunology, Oligonucleotide Synthesis Facility, Rochester, NY). For amplification of germline transcripts (see Fig. 3), a 24-mer sequence from the Iϵ[3] region was used: 5′CGAATAAGAACAGTCTGGC CAGCC3′. This sequence is present only in Iϵ and not in any other Ig sequence (28). For amplification of mature ϵ mRNA (see Figure 3), a set of 4 primers corresponding to the 4 murine JH genes found only in mature Ig transcripts was used:JH1 (24 mer) 5′ GTCTGGGGCGCAGG GACCACGGTC3′;JH2 (24 mer) 5′TACTGGGGCCAAGGCACCA CTCTC3′;JH3 (22 mer) 5′CCAAGGGACTCTGGTCACTGTC3′;JH4 (22 mer) 5′TCAAGGAACCTCAGTCACCGTC3′(37, 38). The 20-mer antisense primer from the sequence specific for exon 1 of Cϵ was used for amplification of both mature and germline ϵ RNA: 5′CTAGGAT AGTCCTACTTTCG3′ (39, 40). The expected PCR product size of germline Cϵ RNA is 320 bp, and the expected size of the amplification of mature Cϵ mRNA is approximately 290 to 310. The size of mature ϵ mRNA is variable because of differences in splice sites, and terminal additions and deletions during the class switching rearrangement of the JH to the C segments (38). Both sets of primers span intron/exon junctions, so that any amplification occurring from contaminating genomic DNA would result in larger-sized products. PCR product sequence was verified by restriction enzyme digests. Actin primer sequences were previously published (41).

RNA preparation and PCR detection of germline and mature ϵ mRNA

Two × 10^7 cells were cultured and total RNA was prepared by lysing cells in 4 M guanidine isothiocyanate, layering the lysate on 5.7 M CsCl, and centrifuging at 100,000 × *g* in a Beckman airfuge (Beckman Instruments, Palo Alto, CA). RNA pellets were precipitated in potassium acetate and ethanol and quantitated by absorbance at 260 nm. RNA was electrophoresed on a 1% formaldehyde-agarose gel to verify integrity. Semi-quantitative PCR was performed using the method of Dallman (34–36). RNA was reverse transcribed and the resulting cDNA was amplified by PCR for 19 cycles, after which PCR product was removed

[3] Abbreviation used in this paper: Iϵ, I region exon sequence present in germline transcripts but not mature ϵ mRNA.

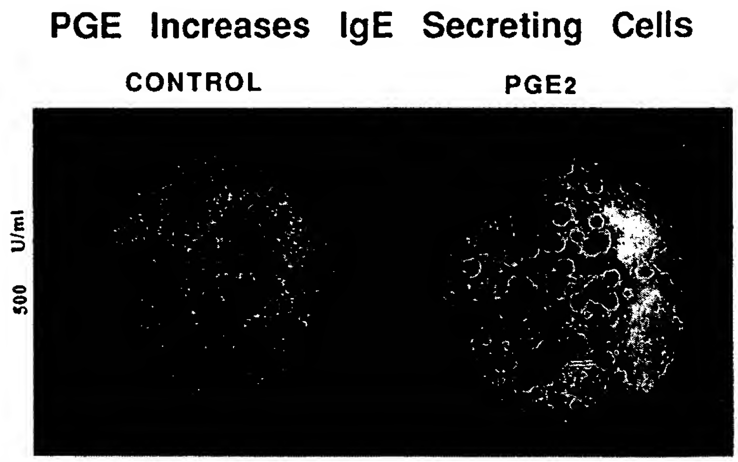

FIGURE 1. PGE increases the frequency of IgE-secreting cells. Resting B cells were incubated ± PGE_2 and stimulated with IL-4 and 5 μg/ml LPS for 5 days. Cells were washed and incubated on anti-IgE coated plates for the IgE ELISA spot as described in *Materials and Methods.*

every three cycles up to 31 cycles. PCR was also performed for β-actin, using previously published primers (41), to confirm equivalent amounts of RNA. To control for the possibility of contaminating genomic DNA, each RNA sample was also PCR-amplified in the absence of reverse transcriptase.

RNA purification and Northern blot analysis

Three $\times$ 10^8 unfractionated B cells were incubated ± PGE_2 10^{-6} M for 18 h, and then stimulated with LPS (5 μg/ml) and IL-4 (500 U/ml) for 48 h. RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH), followed by chloroform extraction, and isopropanol precipitation. Poly(A)$^+$ RNA was isolated using oligo-dT conjugated magnetic beads (Dynal, Oslo, Norway) and was quantitated using an oligo-dT blot technique. Radioactivity was measured on a Matrix 96 Direct β Counter (Packard Instrument Co., Downer's Grove, IL). Equivalent amounts of Poly(A)$^+$ RNA were electrophoresed and transferred to nitrocellulose. The blot was probed with a ^{32}P-labeled Cϵ fragment, generously provided by Dr. Paul Rothman (Columbia University, NY), and exposed to film.

Statistics

ELISA and ELISA spot data are the average of triplicate or quadruplicate cultures. Experiments were repeated three or more times and representative data are shown. Statistical significance was assessed with the two-tailed Student's *t*-test.

Results

PGE_2 increases the number of cells synthesizing IgE

We had shown previously that E series PG significantly increased the amount of IgE in supernatants of unfractionated splenic B cells stimulated with LPS and IL-4, whereas $PGF_{2\alpha}$ had no effect (10, 14, 16, 20). To determine whether PGE increased the number of cells secreting IgE, IgE-secreting cells were enumerated by use of an ELISA-spot assay. Figure 1 shows ELISA-spot wells of B lymphocytes treated with IL-4 and LPS ± PGE_2. PGE clearly increases the number of IgE spot-forming cells (IgE-secreting cells). There is variation in the size of IgE spots reflecting differences in IgE synthetic rate; however, there is no consistent PGE effect on spot size, suggesting that PGE does not increase the rate of IgE synthesis. Table I summarizes the PGE effect on IgE-secreting cell frequency. PGE increased the number of cells synthesizing IgE up to 14-fold in the presence of IL-4 and LPS on all days of IgE secretion, indicating that the PGE effect is not solely due to a kinetic change in the development of IgE-secreting cells. PGE increased the number of IgE-secreting cells at all concentrations of IL-4 tested, although a strict requirement for IL-4 was observed, as no IgE-secreting cells were detected in its absence. Both small dense resting B cells ($p > 1.074$) and large activated B cells ($p > 1.062$) responded to PGE by increasing IgE-secreting cells (Table I). Thus, even though PGE inhibits proliferation by approximately 25%, decreasing the total number of cells (16), it increases the number of IgE-secreting cells. Our research has shown that PGE acts via cAMP signaling in murine B lymphocytes (42). Other agents that increase intracellular cAMP levels, such as dibutyryl cAMP and cholera toxin, also increased the number of cells secreting IgE on all days of IgE synthesis up to 19-fold (data not shown). Spots could not be counted reliably on day 3 after IL-4 and LPS-induced stimulation because the spots formed were very small and ill-defined. On day 7 there were few IgE-secreting cells with no significant difference between PGE and control groups (data not shown).

PGE_2 enhances IgE synthesis in surface IgM positive B cells

Although PGE inhibits LPS driven lymphocyte proliferation by approximately 25% in this system (16), it was possible that PGE was selectively promoting the growth of rare IgE-secreting cells in the splenic population. To determine if PGE was acting on uncommitted surface IgM-positive B cells, or alternatively, was expanding pre-existing IgE-secreting populations, cell-sorting experiments were conducted to isolate B lymphocytes expressing high levels of surface IgM, which are uncommitted pre-switch B cells (24). As shown in Figure 2, in an average of three experiments, PGE enhanced IgE synthesis in surface IgM-positive B cells to a similar extent as in unsorted B cells. Thus, PGE acts on uncommitted B lymphocytes to enhance IgE class switch differentiation.

Table I. *PGE increases the frequency of IgE-secreting B cells*

IL-4 U/ml	Day 4 Control	Day 4 PGE	Day 5 Control	Day 5 PGE	Day 6 Control	Day 6 PGE
Experiment 1[a]						
Resting B cells						
0			1	0	0	0
100	ND	ND	0	5 ± 3	0	2 ± 1
1,000			114 ± 15	636 ± 162[b]	27 ± 6	100 ± 20[b]
5,000			76 ± 1	477 ± 75[b]	51 ± 4	117 ± 20[b]
Activated B cells						
0			2	0	0	0
100	ND	ND	4 ± 3	5 ± 2	0	4 ± 2
1,000			50 ± 10	443 ± 3[b]	31 ± 5	131 ± 19[b]
5,000			58 ± 8	183 ± 17[b]	42 ± 6	192 ± 25[b]
Experiment 2						
Resting B cells						
0	0	0	0	0	0	0
50	1 ± 1	9 ± 8	9 ± 4	38 ± 18	1 ± 0	5 ± 2
100	ND	ND	5 ± 3	69 ± 22[b]	2 ± 2	27 ± 4[b]
1,000	120 ± 33	399 ± 99[b]	210 ± 5	423 ± 43[b]	118 ± 17	329 ± 32[b]
5,000	669 ± 74	1,657 ± 109[b]	593 ± 25	1,943 ± 144[b]	128 ± 7	679 ± 170[b]

[a] B cells (1×10^5) were incubated ± PGE_2 10^{-6} M for 15 h and then stimulated with IL-4 and LPS (5 μg/ml) on day 0. Cultures were incubated for the time shown, and the cells were washed and diluted for enumeration in the ELISA-spot assay described in *Materials and Methods.* IgE-secreting cells could not be counted on day 3 because spots were small and ill-defined, and by day 7, few cells secreted IgE with no significant difference between PGE-treated and control groups.
[b] Groups significantly different from controls $p < 0.05$.

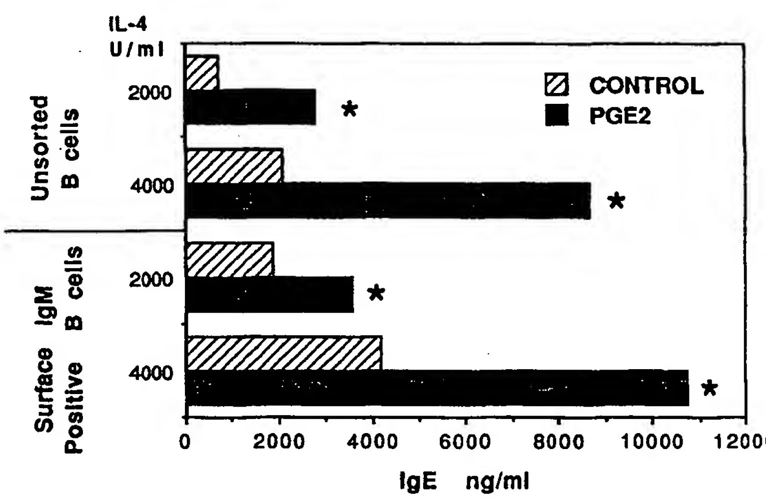

FIGURE 2. PGE promotes IgE synthesis in sorted surface IgM^+ B cells. Resting B cells expressing high levels of IgM were isolated by fluorescence-activated cell sorting. Cells treated for immunofluorescence but not sorted are shown for comparison. Five $\times$ 10^5 B cells were pre-incubated ± PGE_2 at 10^{-6} M for 18 h. Five μg/ml LPS and rIL-4 were then added, and cell supernatants were assayed for Ig 7 days later as described in *Materials and Methods.* Values with PGE_2 are significantly different from controls pre-incubated in media without PG ($p < 0.05$).

We were also interested in determining the effect of PGE on the rate of IgE synthesis in cells that had already switched and were secreting IgE. Two hybridomas, IGELa2 and IGELb4, that secrete IgE were tested for their response to PGE_2 from 10^{-6} to 10^{-8} M in the presence or absence of IL-4 (10–1,000 U/ml) or LPS on days 1 to 3. Under no circumstances did PGE increase IgE synthesis (data not shown) by the cells. This suggests that PGE acts at a point before terminal differentiation, although the regulation of the rate of IgE synthesis in hybridomas may differ from that of naturally occurring IgE-secreting plasma cells.

PGE_2 increases germline ε transcripts

IL-4 induces transcription of the unrearranged ε switch region (germline transcripts) before DNA rearrangement, as depicted in Figure 3*A* (28–31, 33). To determine whether PGE might affect switching by altering transcription through this region, the expression of germline RNA transcripts from the ε switch region were measured. To compare the amounts of rare ε transcripts (the absolute highest frequency of cells induced to secrete IgE was 2%), we employed the powerful technique of semi-quantitative PCR (34–36). In this technique, the relative amount of RNA (reverse transcribed into cDNA) present in samples

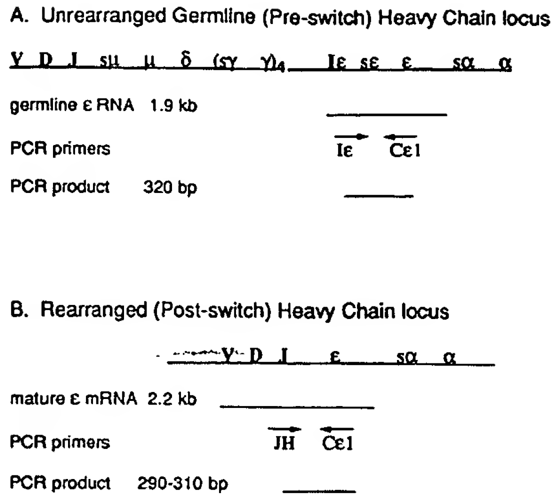

FIGURE 3. Unrearranged germline (*A*) and rearranged (*B*) heavy chain locus. The positions and 5′ to 3′ orientations (arrows) of primers are shown. Iε primer is specific for germline ε RNA, J_H primers are specific for H chain mature Ig mRNA, and Cε1 is specific for both ε RNA.

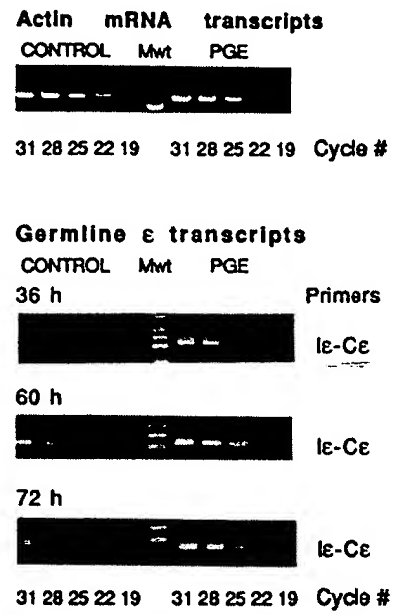

FIGURE 4. PGE increases germline ε RNA. Resting B cells were treated ± PGE_2 at 10^{-6} M for 15 h and then stimulated with IL-4 (1000 U/ml) and LPS (5 μg/ml). Germline ε transcripts (approx 0.3 μg RNA) were amplified by PCR using 5′ primer specific for Iε sequence found only in germline transcripts and a 3′ primer sequence specific for Cε. PCR product was removed every 3 cycles beginning at cycle 19. PCR amplification products using primers specific for actin mRNA (0.1 μg) are shown for comparison.

can be determined by analyzing PCR products as they accumulate during PCR cycles before the plateau phase of the reaction. If more specific RNA is present in a sample, more PCR product will be amplified, in a given number of PCR cycles, compared with a sample with less of the specific RNA. To specifically amplify germline transcripts, a primer specific for the Iε exon (present only in the germline ε RNA, and deleted during IgE class switching) (28, 37) was used in conjunction with a sequence complementary to exon 1 of Cε (39, 40).

As shown in Figure 4, germline ε RNA transcripts were detected at earlier PCR amplification cycle numbers in PGE-treated groups. PGE increases the amount of germline ε RNA 36–96 h after stimulation with IL-4 and LPS, although actin mRNA levels were very similar. At 36 h, PCR product is visible in ethidium-stained gels under ultraviolet light after 22 cycles in the PGE-treated group. In contrast, the control group PCR product is visible only after 25 cycles and remains diminished relative to the PGE- treated group up to 31 cycles. Theoretically, if there is the same amount of PCR product in the control group after 25 cycles and in the PGE-treated group after 22 cycles, then there is eight (2^3) times more PCR product in the group treated with PGE compared with the control, because each PCR cycle doubles the reaction product (35). Densitometric scanning of photographic negatives of ethidium-stained gels (36 to 72 h), showed an increase of germline ε RNA PCR product of 4 to 10-fold in the presence of PGE when compared with controls (normalized to actin PCR product). Germline ε RNA transcripts were readily detectable 24 h after IL-4 and LPS stimulation (data not shown); however, no stimulation of germline ε RNA transcripts by PGE could be detected at early time points, possibly because of PGE inhibition of early B cell activation events (10). Expression of germline ε RNA transcripts showed a strict requirement for IL-4; no germline ε transcripts could be detected from even 3 μg of RNA from B cells treated with LPS ± PGE in the absence of IL-4.

To confirm the PCR results, poly(A)$^+$ RNA from cells treated ± PGE_2 and IL-4 and LPS for 48 h was analyzed by Northern Blot. As shown in Figure 5, PGE increased the steady-state level of the 1.9 Kb germline ε transcript (fourfold measured by densitometry) compared with controls stimulated with IL-4 and LPS alone.

PGE_2 increases mature ε mRNA transcripts

To follow the process of Ig class switching ± PGE, post-switching mature V(D)J ε mRNA was also specifically measured by semi-quantitative PCR (34–36). As shown in Figure 3*B*, a mixture of four primers specific for the four murine JH genes (present only in mature Ig mRNA) was chosen to amplify the 5′ end of the mRNA. The 3′ primer was the same Cε exon 1 sequence for both germline and mature ε transcripts as described in *Materials and Methods*. As shown in Figure 6, PGE induces mature ε mRNA

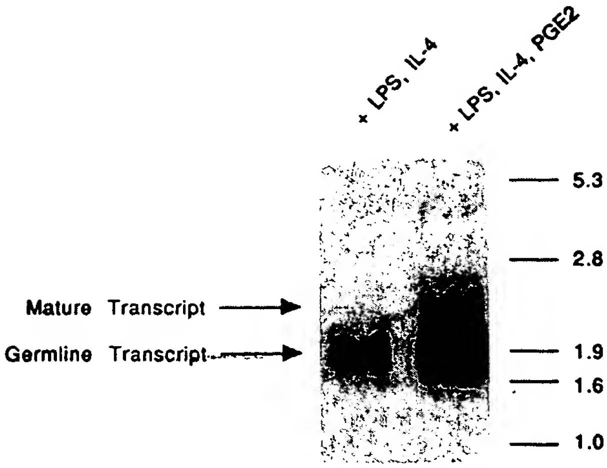

FIGURE 5. PGE increases expression of germline and mature ϵ mRNA. (Northern analysis). Three $\times$ 10^8 B cells were incubated $\pm$ PGE_2 10^{-6} M for 18 h and then with 500 U/ml IL-4 and 5 μg/ml LPS for 48 h.

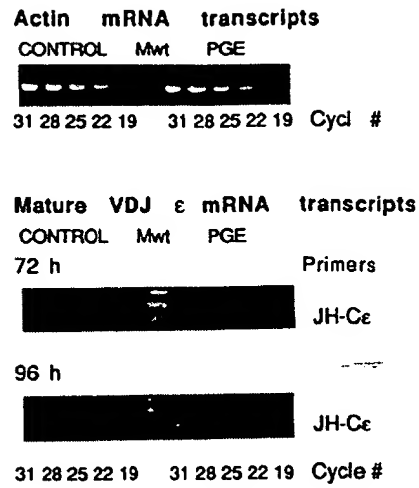

FIGURE 6. PGE increases expression of mature V(D)J ϵ mRNA. Resting B cells were treated $\pm$ PGE_2 at 10^{-6} M for 15 h and then stimulated with IL-4 (1000 U/ml) and LPS (5 μg/ml). Mature V(D)J ϵ mRNA transcripts (1 μg RNA at 72 h, 0.6 μg 96 h) were amplified by PCR using a set of 5′ primers specific for the four murine J_H sequences and a 3′ primer sequence specific for Cϵ. PCR product was removed every 3 cycles beginning at cycle 19. PCR amplification products using primers specific for actin mRNA (0.1 μg) are shown for comparison.

transcripts 72 h after IL-4 and LPS-induced stimulation, whereas transcripts are not detected from the same amount of RNA (1 μg) from cells treated with IL-4 and LPS alone. In the presence of PGE, mature ϵ mRNA is detected by PCR after 25 cycles, but the message cannot be detected after even 31 cycles in the control (IL-4, LPS, no PGE). For comparison, actin mRNA was amplified to very similar levels in control and PGE-treated cells at both time points. Densitometric scanning of photographic negatives of the 72-h ethidium-stained gel showed a 10-fold increase in mature ϵ mRNA PCR product in the presence of PGE as compared with controls (normalized to actin PCR product). At 96 h, mature ϵ mRNA remains elevated in PGE-treated cells, correlating with an increase in the number of cells secreting IgE on day 4 (Table I). Mature V(D)J ϵ mRNA was not detected in the absence of IL-4.

Northern analysis (Fig. 5) also showed a 10-fold increase in the 2.2 Kb mature ϵ mRNA when PGE_2 is present in addition to IL-4 and LPS. These data indicate that PGE not only increases germline ϵ RNA transcripts, but also promotes the switch to expression of mature ϵ mRNA.

Discussion

PGE increased the number of splenic B cells secreting IgE as measured by ELISA spot assay. Since PGE inhibits IL-4 and LPS-induced B cell proliferation by 25% (16), the enhancement of IgE synthesis is even more dramatic on a per cell basis. A maximum frequency of approximately 2% (2,000 of 100,000 B cells) could be stimulated to secrete IgE in the presence of PGE, IL-4 (5,000 U/ml) and LPS. PGE increased the number of IgE-secreting cells on all days of measurable IgE production, ruling out a simple kinetic effect. Furthermore, three lines of evidence suggest that PGE does not enhance the rate of IgE synthesis: 1) There was no discernible difference in the size of the spots that developed in the IgE ELISA $\pm$ PGE (Fig. 1); 2) PGE enhancement of supernatant IgE levels paralleled the increase in the number of cells secreting IgE with PGE at a given IL-4 concentration (Table I, Figure 2); and 3) PGE did not increase IgE synthesis by IgE-secreting hybridomas. Therefore, PGE acts mainly by increasing the number of cells that produce IgE in response to IL-4 and LPS.

To investigate the possibility that PGE was selectively enhancing the outgrowth of a population of cells that were precommitted to IgE-secretion, we determined that PGE acts on surface IgM-positive B lymphocytes, which are uncommitted B cells that do not express IgE (24). Our results demonstrate that PGE can synergize with IL-4 to direct the isotype commitment of a B cell to Cϵ synthesis and as such can determine the type of effector B cell that develops during an immune response. Interestingly, surface IgM-positive sorted cells tended to produce slightly higher levels of IgE than cells similarly treated but not sorted. It is possible that a rare contaminating inhibitory cell type may be removed by sorting, or B lymphocytes that express high levels of IgM may be intrinsically more disposed to switching or more responsive to the effects of IL-4 and LPS.

Because PGE acts on uncommitted B cells and increases the number of cells secreting IgE, we hypothesized that PGE was enhancing classical B cell isotype switching (depicted in Figure 3) to IgE secretion with IL-4 and LPS. Similar to IL-4, PGE may affect chromatin structure and

enhance the accessibility of the ϵ switch region to nuclear enzymes, including the switch recombinase, and thereby aid in directing the isotype switch to IgE (33). Alternative explanations for increased IgE production include PGE induction of long transcripts from the variable region through an upstream CH gene (e.g., $\gamma 1$) and the $C\epsilon$ region with alternate splicing yielding V(D)J ϵ mRNA, analogous to IgD production. This mechanism has been invoked for expression of IgE, but remains controversial (29, 31, 43). Another possibility is that PGE promotes RNA trans-splicing between mRNA for V(D)J IgM/IgD and germline ϵ transcripts, generating a V(D)J ϵ mRNA hybrid transcript, similar to the Ig gene trans-splicing mechanisms described in murine B cell leukemia clones and in transgenic B cells (44, 45). However, if the increase in IgE synthesis were resultant from trans-splicing, it should be possible for these hybrid transcripts to form as soon as both transcripts were present in the cell. The fact that V(D)J IgM/IgD is already expressed in unstimulated B cells and that germline ϵ transcripts are present the first day after IL-4 and LPS-induced stimulation, but that V(D)J ϵ mature transcripts are only detectable after day 3, argues against this mechanism of IgE synthesis. A caveat is that PGE might activate a trans-splicing mechanism 3 days after cell stimulation.

PCR and Northern analysis in this manuscript demonstrate that PGE affects early B lymphocyte differentiation before the H chain isotype switch recombination event has occurred, acting on surface IgM positive B cells to increase germline ϵ switch region transcripts in IL-4 and LPS-stimulated B cells. The fact that mature V(D)J ϵ mRNA transcripts arise earlier with PGE than in cells stimulated with IL-4 and LPS alone indicates that PGE not only augments germline transcripts, but also promotes the class switch to IgE expression. Thus, similar to IL-4, PGE promotes class switching to IgE in B lymphocytes stimulated with IL-4 and LPS. Underscoring the complex regulatory nature of PGE, we found that although PGE enhances IgE class switch differentiation days after stimulation, PGE inhibits certain early activation events in the same population of B cells (10, 42). This fact may explain why clear PGE enhancement of germline transcripts was not seen at 24 h, but only at later times when PGE inhibitory effects wane (10).

The action of PGE in B lymphocytes is cAMP-dependent (14, 16, 20, 42). Interestingly, agents that induce cAMP increase V(D)J recombinase activity (46), suggesting the possibility that cAMP-inducing agents (including PGE) might also regulate the activity of the heavy chain class switch recombinase. If this were the case, PGE might be expected to increase switching to all isotypes. PGE is known to increase IgE, IgG1, IgG2a, whereas other agents that increase cAMP enhance switching to IgE, IgG1, IgG2a, and IgA (10, 14, 16, 19, 47). PGE decreases IgG3 synthesis (16), but this may be a result of either decreased switching to IgG3 or increased switching from IgG3 (the most 5′ $C\gamma$) to further downstream CH genes. We have identified by 2D gel electrophoresis three proteins induced by PGE and other cAMP-inducing agents (42). These PGE-inducible proteins may act to regulate B cell activation and differentiation. Further research will be required to determine whether PGE promotes class switching by mechanisms in addition to the enhancement of germline transcripts.

We are particularly interested in PGE regulation of IgE production because of its importance in human disease. Cross-linking of IgE bound to mast cell and basophil Fc receptors is responsible for Type I hypersensitivity, and plays a major role in the pathogenesis of certain diseases, such as the hyper-IgE syndrome. Interestingly, the monocytes of hyper-IgE patients constitutively secrete high quantities of PGE (48). A correlation between overproduction of PGE and increased IgE synthesis can also be seen in rheumatoid arthritis and Hodgkin's lymphoma (16). Furthermore, high levels (400% of controls) of PGE are secreted by monocytes from trauma patients (49, 50). Significantly, these patients mirror our in vitro system, developing elevated serum IgE and markedly depressed IgM levels (16, 51). A similar scenario exists in a septic rat model and in septic patients: PGE secretion by monocytes and macrophages is increased, PGE levels in serum are elevated, and IgE is significantly increased, whereas plasma IgM levels are lowered (50–52). Moreover, PGE could promote IgE production in vivo because PGE inhibits T lymphocyte secretion of IFN-γ which blocks IgE synthesis, while sparing lymphokines that promote switching and IgE synthesis (IL-4 and IL-5) (20). In a person sensitized to one allergen, cAMP-inducing agents released during each allergic episode (e.g., PG and histamine) (53, 54) may feed back into the system and promote B cell isotype switching to IgE, thus contributing to the allergic subject's tendency to rapidly become hypersensitive to a host of Ags. In conclusion, consistent with a role for PGE inducing class switching to IgE in vivo, an array of clinical conditions exists in which overproduction of PGE is associated with promotion of IgE synthesis. Although the regulation of IgE synthesis is complex, it may be possible to reduce IgE levels in certain clinical conditions by interfering with PG synthesis.

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